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THE UNIVERSITY OF ALBERTA

PURIFICATION AND CHARACTERIZATION
OF A HUMAN
ADENOVIRUS-ASSOCIATED
ENDONUCLEASE-LIKE ACTIVITY

by

© LINDA WAI-LING TSANG

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

IN

VIROLOGY

DEPARTMENT OF MEDICAL BACTERIOLOGY

EDMONTON, ALBERTA

FALL, 1978

To

My Beloved Mother

ABSTRACT

Human adenovirus-associated endonuclease-like (AAEL-) activity was purified from adenovirus type 5 virions or excess pool soluble components by successive steps of centrifugation and column chromatography. The endonuclease-like activity was obtained in a form free from, and in association with, the penton structural component. SDS-polyacrylamide gel analysis of free-aggregated AAEL-activity showed that the enzymatic-like activity was associated with a polypeptide of 32,000-32,500 daltons.

Purified AAEL-activity was found to be stable at room temperature, require specific ions for activity and not to be affected by SDS at concentrations up to 5%. A proteinaceous, Pronase-resistant, AAEL-activity inhibitor found in association with crude preparations was shown to be labile to pH and room temperature storage.

AAEL-activity DNA substrate (homologous adenovirus DNA or phage PM2 DNA) cleavage patterns, as analyzed by agarose gel electrophoresis, were found to differ at pH 4.5 and pH 7.4, thus indicating the presence of different conformational states of the AAEL-activity under different physical conditions.

AAEL-activity appeared to recognize a particular substrate DNA sequence, close to the 3' or 5' terminal (replication initiation site ?). This particular sequence was also found in human adenovirus type 2 DNA or phage PM2 DNA. Size of the AAEL-activity cleavage products of adenovirus type 5 DNA varied from slightly larger than the Eco RI restriction enzyme B fragment to smaller than the Eco RI C fragment.

Temperature-sensitive, conditional-lethal mutants of adenovirus types 2 and 5, and selected recombinants of such, were used in conjunction with specific antisera against AAEL-activity, virion and structural components, to show that the AAEL-activity is most likely host-cell derived, virion-induced and modified and virion-incorporated.

To see a World in a Grain of Sand
And a Heaven in a Wild Flower,
Hold Infinity in the palm of your hand
And Eternity in an hour.

Auguries of Innocence
William Blake

ACKNOWLEDGMENTS

The author wishes to express her appreciation to Dr. R. G. Marusyk for his guidance, encouragement and patience during the course of this study. She is grateful to Dr. G. Wadell and Dr. J. Weber for the provision of the viruses.

Special thanks to Mrs. H. Marusyk, Mr. N. Chang, Mr. T. Chui and Mr. P. Fedorak, and members of the Department of Medical Bacteriology for their fortitude and assistance.

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ABBREVIATIONS

CPE	cytopathic effect
DEAE	diethylaminoethyl
EDTA	(ethylenedinitrilo) tetraacetic acid, disodium salt
FU	fluorescence unit
g	gravity (centrifugal force)
h	hour
HAd	human adenovirus
HE	hemagglutination enhancement
M	molar
MW	molecular weight
mM	millimolar
MEM	minimum essential medium
min	minute
ug	microgram
ul	microliter
OD	optical density
nm	nanometer
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffer saline
PEG	polyethylene glycol

PFU	plague forming unit
PI	post infection
SDS	sodium dodecyl sulphate
SSC	saline sodium citrate
Tris	tris (hydroxymethy) aminomethane
w/v	weight per volume

INTRODUCTION

In 1953, Rowe and colleaques observed cytopathic changes in cultures derived from adenoid tissues used for the isolation of an elusive respiratory virus. In the following year, Hilleman and Werner (1954) isolated several similar cytopathic agents in cultures of human tissues during a study of epidemics of an influenza-like disease in army recruits. These new agents were named adenovirus by Enders et al. (1956) to record the original isolation from adenoid tissues.

The adenovirus is non-enveloped with a diameter ranging from 65-80 nm. The virion consists of a central nucleoprotein core and an outer capsid. The core has a diameter of 40-45 nm and contains viral DNA with a molecular weight of approximately 23×10^6, and 20% of the total viral protein. The capsid is composed of 252 polygonal capsomeres arranged in icosahedral symmetry. Of the 252 capsomeres, 240 have six neighbours and are known as hexons (Ginsberg et al., 1966). The remaining twelve capsomeres, situated at the vertices of the icosahedron, have five neighbours and are known as pentons (Ginsberg et al., 1966; Valentine & Pereira, 1965). The penton has two parts - the penton base and fiber - which are linked together by non-covalent bonds

(Pettersson and Hoglund, 1969). These subunits have been isolated, purified and characterized from infected cells (Pettersson et al., 1967, 1968, Petterson & Hoglund, 1969, Petterson, 1971) in order to investigate the possible individual role of each as complete virions assemble.

Certain viruses can agglutinate red blood cells due to the interaction of viral surface antigens and receptor sites on the cell membrane. The ability of adenovirus to hemagglutinate was first demonstrated by Rosen (1958). Subsequently, all human adenovirus serotypes have been shown to display this characteristic (Rosen, 1960; Rosen et al., 1962; Schmidt et al., 1965). Morphological studies of isolated subunits of adenovirus have shown that the type specific determinant "gamma", located at the distal end of the fiber, can interact with specific receptors on the surface of red blood cells (Pereira & Figueiredo, 1962; Valentine & Pereira, 1965; Norrby, 1966; Pettersson et al., 1968; Norrby, 1969). In 1960, Rosen proposed a subgroup classification of the human adenoviruses based on their ability to agglutinate different species of red blood cells. Subgroup I viruses can completely agglutinate rhesus monkey erythrocytes, subgroup II completely agglutinate rat cells and subgroup III partially agglutinate rat cells.

In productive adenovirus infection, cytopathic changes in cell cultures can be observed as early as 8-24 hours post infection. The early cytopathic factor or toxin effect was found to be associated with penton components (Valentine & Pereira, 1965), specifically the penton base (Pettersson and Hoglund, 1969; Winters et al., 1970). Aside from causing cytopathic changes in cells, an endonuclease was also reported to be associated with this subunit by various investigators (Burlingham & Doerfler, 1971a; Burlingham & Doerfler, 1972; Marusyk et al., 1975; Medveczky et al., 1976; Reif et al., 1977 a,b).

The association of enzymes with viral components has been shown to exist in many virus groups. In 1943, Hirst reported the first virus-associated enzyme - the neuraminidase of the orthomyxovirus group. Since then, several enzymes existing within viruses have been reported. In reovirus, an RNA transcriptase has been detected in association with the double-stranded genome (Shatkin & Sipe, 1968). Besides a nucleotide phosphohydrolase, frog virus 3 also carries endodeoxyribonuclease and endoribonuclease activities within the virion, detectable only after detergent treatment of the virion (Kang & McAuslan, 1972). In the poxvirus group, DNA-dependent RNA polymerase (Kates & McAuslan, 1967; Munyon et al., 1967), phosphohydrolase (Gold

& Dales, 1968; Munyon et al., 1968), and two deoxyribonucleases were reported in association with the virus core (Pogo & Dales, 1969).

Endonuclease activity has been detected in association with polyoma and SV40 virus after several cycles of isopynic centrifugation (Parodi et al., 1974; Rouget et al., 1976) and has properties different from that of the calf serum endonuclease reported by McMillen et al., (1976) and Dooley et al., (1976). The endonuclease cleavage site is located in the A-T rich region at a position opposite to the origin of replication in both SV40 and polyoma virus genomes. From studies of a thermosensitive mutant (ts A) of polyoma virus, it was shown that the viral genome could code for a site specific endonuclease which is involved in the control of DNA replication as well as the integration and excision process (Stocker & Dulbecco, 1969; Cuzin et al., 1970). The papova virus-associated endonuclease might, therefore, play a role in viral genome replication and/or transformation (Cuzin et al., 1971, 1973, Rouget et al., 1976).

Three size-classes of intracellular DNA were detected following rate zonal sedimentation of cell extracts productively infected by adenovirus type 2 (HAd2) or HAd12, or abortively infected by HAd12 (Burlingham & Doerfler,

1971b). The three size classes were as follows:

(I) Intact, free viral DNA with a sedimentation rate of 31.3 S for HAd2 and 29.0 S for HAd12.

(II) DNA sedimenting at a rate greater than 45 S. Apparently, the viral DNA was integrated into fast-sedimenting cellular DNA

(III) fragments of viral DNA of approximately 5 x 10^6 daltons sedimenting at 18 S.

Cellular DNA was apparently cleaved even in the absence of DNA, RNA and protein synthesis (via the use of chemical inhibitors). Therefore, it was suspected that a pre-existing enzyme was present in the cells (Burlingham & Doerfler, 1971b).

An endonuclease specific for double-stranded DNA was subsequently found in association with the pentons of HAd2 as well as in intact virions (Burlingham & Doefler, 1971). In the study of Marusyk and colleaques (1975), endonuclease activity was detected only in disrupted and not intact virions.

By selective digestion of the penton base with trypsin (Burlingham & Doerfler, 1971), the endonuclease activity was

destroyed. Furthermore, Burlingham & Doerfler found that the amount of activity present was related to the amount of penton base component rather than the amount of protein present. Antiserum prepared against the penton base could inhibit the endonuclease activity. The above evidence gave strong support for association of endonuclease activity with the penton base.

In extracts of cells productively or abortively infected with HAd2 or HAd12, endonuclease activity was detected 2 - 8 hours postinfection, co-inciding with the uncoating of the virus. During productive infection, an increase of activity was observed to occur simultaneously with the synthesis of viral structural proteins (Burlingham & Doerfler, 1972).

Further evidence for the existence of adenovirus-associated endonuclease activity was reported by Marusyk & Norrby (1971). In studies of two canine adenoviruses (Marusyk & Norrby, 1971), excessive amounts of penton material were found in association with intact virions at a buoyant density of 1.34 g/ml following CsCl centrifugation. Pentons, being entirely protein in nature (Pettersson & Hoglund, 1969), would be expected to display a buoyant density of 1.30 g/ml. Electron microscopic examination

revealed non-symmetrical groupings of pentons, aggregated at the vertex capsomeres, together with intact virions. When the high density pentons were treated with nuclease, a buoyant density shift to about 1.31 g/ml was observed. These results indicated the presence of a segment of DNA-like material attached to the pentons, resulting in an increase in buoyant density. The high density penton structures were postulated to be a complex of penton, endonuclease and DNA.

Endonuclease activity causing single strand-scissions in substrate DNA has been detected in association with highly purified virion-derived or pooled pentons and dodecons of representatives from all three subgroups of human adenoviruses (Marusyk et al., 1975; Medveczky et al., 1976)

Two endonucleases isolated from HAd2-infected and uninfected cells were separated and characterized by Rief et al. (1977 a,b). Both endonucleases create single strand 'nicks' but at different optimal pH. The type of cleavage detected indicated that there was no apparent degree of specificity or that the substrate sites recognized occurred very frequently.

The biological function of adenovirus-associated endonuclease activity is not yet known. The endonuclease

activity may play a role in replication of the viral genome or in recombinational events occurring between viral and cellular DNA.

Shortly after adenovirus infection, several viral specific DNA-binding proteins of molecular weight 11,000 to 75,000 are synthesized (Russell & Skehel, 1972; Van der Vliet & Levine, 1973; Walter & Maizel, 1974; Gilead et al., 1976; Harter et al., 1976; Saborio & Oberg, 1976; Levinson & Levine, 1977). The 75,000 protein coded for by the HAd2 genome at a site located between 0.62 and 0.68 on the genome physical map (Grodzicker et al., 1974) has been of great interest because it binds specifically to single stranded DNA (Van der Vliet & Levine, 1973; Shanmugan et al., 1975), and is often found in association with a 45,000 dalton protein, probably a breakdown product of the 75,000 protein (Rosenwirth et al., 1975; Levinson & Levine, 1977), in a membrane associated replication complex isolated from infected cells. Both the 75,000 and 45,000 protein were associated with newly synthesized DNA and a DNA polymerase activity (Yamashita & Green, 1974; Yamashita et al., 1975; Gilead et al., 1975). Studies of thermolabile mutants of HAd5 indicated that the DNA binding protein might play a role in HAd DNA replication (Levine et al., 1974: Van der Vliet, et al., 1975). This hypothesis was further supported

by the isolation of a tumor antigen with similar DNA binding properties and size in the SV40 systems.

The adenovirus core contains DNA and two proteins, polypeptides V (48,500) and VII (18,000) (Everitt et al., 1973; Everitt & Philipson, 1974; Laver, et al., 1968; Laver, 1970). Polypeptide VII is tightly bound to DNA and is covered by an outer protein shell probably formed by polypeptide V , as revealed by electron microscopic study of sarcosyl-treated HAd2 virions (Brown et al., 1975). Isolated DNA from the sarcosyl core was circular in structure and was susceptible to DNase treatment. Following Eco RI restriction enzyme cleavage and pronase digestion, six specific DNA fragments were resolved on polyacrylamide agarose gels. However, with the omission of Pronase digestion, a complex between Eco RI fragments A and C and protein was isolated. The protein apparently forms a "bridge" between the ends of the viral DNA and presumably is responsible for the circularization of the viral DNA inside the core (Robinson et al., 1973; Robinson & Bellet, 1974; Doerfler et al., 1974; Brown et al., 1975; Corden et al., 1976).

In the study of DNA-binding protein associating with the ends of intact native HAd DNA, Rekosh et al. (1977) found that the protein comprised only 0.1 % of the DNA-

protein complex, similar to results obtained with an avian adenovirus (Robinson & Bellet, 1974) and resulted in a shift in buoyant density of only 0.002 ± 0.001 g/ml as detected by analytical ultracentrifugation analysis. The molecular weight of the protein was estimated by SDS PAGE gels to be 55,000. The protein is bound tightly to DNA, possibly via a covalent linkage (Rekosh et al., 1977).

The 55,000 DNA-binding protein might function in protecting the DNA from exonuclease digestion (Sharp et al., 1976) or act as an endonuclease (Cavalier-Smith, 1974; Tattersall & Ward, 1976) in the initiation and completion of the 5' ends of progeny DNA strands (Robinson & Bellet, 1974).

In 1977, a model of adenovirus DNA replication was proposed by Rekosh et al in which the binding protein plays a significant role. The protein might function as a nuclease-ligase complex in which catalytic subunits might be present and have the capability of cleaving and linking DNA strands covalently (Robinson & Bellet, 1974).

Several proteins have been found to attach to nucleic acid in different animal viruses (Kasamatsu & Wu, 1976; Lee et al., 1977), bacterial plasmids (Blair et al., 1971; Blair & Helinski, 1975; Lovett & Helinski, 1975; Guiney &

Helinski, 1975), and bacteriophages (Ortin et al., 1971; Arnberg & Arwent, 1976). These proteins were found at or near the origin of nucleic acid replication in studies where the origin was located. It is possible, therefore, that the protein(s) has some role in the initiation of nucleic acid replication.

The above review indicates that certain proteins associating with different viral DNAs may exhibit endonuclease-like activity, cleaving double or single-stranded DNA either specifically or non-specifically. The following study was undertaken to isolate, separate and characterize proteins demonstrating endonuclease-like activity in association with soluble components and virions of HAd5.

MATERIALS AND METHODS

1 Cell cultures

HEp-2 cells were obtained commercially (Flow Laboratories, Inc., Inglewood, Ca.). Cells were grown in glass culture vessels (Blake bottles) and when 90% cell confluency was reached, medium was removed, cells washed with 2 ml 0.25% (w/v) trypsin (Difco) and a further 2 ml of trypsin (concentration as above) added and incubated at room temperature for a sufficient amount of time for detachment of cells from the glass substrate. Cells were diluted to approximately 1×10^6 cells/ml in 100 ml of medium and re-dispensed into culture vessels. When the cell monolayer reached 75% confluency (24-48 h), cells were either infected with virus or further incubated at 37°C until repassage.

2 Cell culture medium

Minimum essential medium (MEM; Eagle's modified Auto-POW, Flow Laboratories, Inc., Inglewood, Ca.) was dissolved in distilled water and autoclaved for 15 min. at 15 psi, 120°C. Prior to use, medium was supplemented with 3% calf serum. Glutamine and 7.5% sodium bicarbonate were added to a

final concentration of 0.002 M and 0.1% (w/v) respectively. To each litre of medium, 10 ml of antibiotic solution (final concentration 100 units/ml penicillin-G, 200 ug/ml streptomycin sulfate) was added before use.

3 Virus isolates

Human adenovirus serotypes 2 and 5 (HAd2, HAd5) were the kind gift of Dr. G. Wadell, Dept. of Virology, Karolinska Institute. Stockholm, Sweden. Human adenovirus temperature-sensitive mutants HAd2 tsl, HAd2 ts3, HAd2 ts4, HAd2 ts48 (assembling mutants) and HAd5 ts36 (DNA minus at non-permissive temperature) were gifts from Dr. J. Weber (Dept de Microbiologie, Universite de Sherbrooke.)

4 Virus propagation

Ten Blake bottles at 75% cell confluency were routinely used for virus propagation. Before infecting, tissue culture medium was discarded and cells were adsorbed with 10 ml of purified virus suspension (10 PFU/cell) in phosphate buffered saline (PBS) for 1 h at 37°C with occasional swirling of the virus suspension over the cell monolayer. After the adsorption period, 70 ml of medium was added and the virus-infected cells were further incubated at 37°C for

48-72 h. When complete cytopathic effect was obtained, media containing the infected cells were pooled and centrifuged at 800 xg for 15 min. The cell pellet was suspended in PBS or 0.02 M Tris-HCl, pH 9.0, and stored at -20°C until used.

5 Purification of virus

Two methods were utilized for virus purification:

a) Fluorocarbon extraction: The frozen virus-infected cell pellet was suspended in a mixture of 1 part Freon 113 (Dupont, Maitland, Ontario) and 2 parts PBS and homogenized in a Sorvall Omni-Mixer (Dupont Instruments, Wilmington, Delaware) over a period of 4 min with 2 min of cooling between each minute of mixing. Cell debris was removed by centrifugation at 800 xg and the supernatant was layered on a preformed gradient of CsCl consisting of 5 ml of each of the following buoyant densities: 1.40 g/ml, 1.32 g/ml and 1.20 g/ml. Centrifugation was done at 70,000 xg for 90 min in a Beckman L5-50 ultracentrifuge (Beckman Insts., Palo Alto, CA.) using a SW27 rotor. The region above the opalescent band of virus was pooled and stored at -20°C as "soluble components". The complete band of virus

was removed and further purified by equilibrium centrifugation in CsCl, buoyant density 1.339 g/ml, at 100,000 xg in a Beckman SW50 rotor for 18-20 h at 4°C.

b) The frozen pellet was suspended in 0.2% (w/v) sodium deoxycholate (Fisher Scientific Co., Fair Lawn, N.J.) in 0.02 M Tris-HCl, pH 9.0, and mixed, with continuous shaking, at room temperature for 60 min followed by virus purification in CsCl as described above.

6 Virus assay

Viral infectivity was determined by plaque assay using a modification of the technique described by Williams (1970). HEp-2 cells at a concentration of 1×10^6 cells/ml were seeded into disposable tissue culture dishes (60 mm diameter) and maintained in medium at 37°C in a 5% carbon dioxide atmosphere. The cell monolayers were washed twice with 5 ml of PBS and 0.3 ml of each of a ten-fold serial dilution series (10^{-3} - 10^{-11}) of purified virus was inoculated into duplicate cultures, with two uninoculated cultures serving as controls. The plates were rotated gently at 15 min intervals throughout the adsorption period of 60

min at 37°C. After the adsorption period, the cell monolayers were overlaid with melted agar medium prepared as follows:

A= 1.8% Noble agar (Difco) in distilled water, autoclaved and kept at 43°C.

B= 2X MEM, without phenol red, containing 2X concentration of medium additives and magnesium chloride added to a final concentration of 25 mM, kept at 37°C.

The cell monolayers were overlaid with 5 ml of 1 part A and 1 part B agar medium. After the agar had solidified, all plates were inverted and incubated at 37°C in an atmosphere of 5% carbon dioxide. Four days post-infection (PI), 3 ml of similar overlay were added, and on the seventh day PI, a third overlay containing 1:10,000 neutral red was added. Plates were further incubated for 24-48 h and examined for the development of plaques.

7 Preparation of hyperimmune serum

Purified adenovirus was obtained as described above. Spectrophotometric measurment was utilized for protein determination (1 OD at 260 nm is equivalent to approximately 0.283 mg viral protein; Dr. R. Marusyk, personal

communication). Purified virus (1 mg/0.5 ml) was dialysed against PBS at 4°C for 3 h and mixed with an equal volume of complete Freund's adjuvant and injected into rabbits intramuscularly. A 1 ml (1 mg/ml) booster of purified virus was injected intravenously one month after the first injection. Ten days after the last injection the animals were exanguinated, sera collected and inactivated at 56°C for 30 min, then stored at -35°C until required.

8 Hemagglutination-enhancement assay

Serial two-fold dilutions of virus or soluble components were prepared in PBS using a microtitration system (Cooke Engineering Company, Alexandria, Va., U.S.A.). To each 25 ul of diluted antigen, an equal volume of HAd6 antiserum was added and incubated at 37°C for 30 min. Fifty ul of 1% rat erythrocytes in PBS were then added to each well and the mixture further incubated at 37°C for 1 h. The hemagglutination-enhancement titer was taken as the highest dilution of antigen (plus enhancing antibody) which produced complete agglutination of erythrocytes.

9 Detection of AAEL-activity

Purified virus, prepared as described above was

dialysed versus distilled water for 60 min, followed by dialysis against 0.005 M Tris-maleate buffer, pH 6.2 (TMB), for 20 h at 4°C. The dialysate was submitted to centrifugation at 80,000 xg for 2 h and the supernatant subsequently used as a source of virion-derived AAEL-activity. Endonuclease activity was measured by the fluorimetric assay procedure.

Controls consisted of noninfected cells extracted as in the virus purification procedure, and centrifuged at 70,000 xg for 90 min. The fluorimetric assay procedure was utililized for the detection of cell-associated endonuclease-like activity.

10 Fluorimetric assay

The incubation mixture for the assay consisted of 0.01 M citrate, pH 4.5, or 0.01 M Tris-HCl, pH 7.4, plus 0.01 M NaCl, 0.002 M magnesium chloride, 5 ug PM2 DNA and a 10 ul aliquot of the test material in a total volume of 100 ul. The reaction mixture was incubated at 37°C, and a 10 ul aliquot removed at appropriate intervals and added to a standard fluorescing solution consisting of 0.02 M potassium phosphate buffer, pH 12, 0.0002 M EDTA, 0.5 ug/ml ethidium bromide (EB solution; Morgan & Pulleyblank, 1974). The

fluorescence was determined relative to a blank, with excitation at 525 nm and emission at 600 nm in a Turner 430 Spectrofluorimeter. The fluorimetric standard consisted of 10 ul of calf thymus DNA solution (1 OD260/ml) in 2 ml of EB solution. For heat treatment, the solution was heated to 98°C for 2-3 min and cooled to room temperature. All fluorimetric readings were at 23°C.

11 Iodination procedure

Constituents of the reaction mixture were added in the following order: 10 ul 0.1 N HCl; 10 ul 1.0 M PO4, pH 7.0; ^{125}I in 0.1 N NaOH (0.2-1.0 mCi); 50 ul viral protein (250 ug); 25 ul Chloramine T (0.2 mg/ml). The mixture was incubated at room temperature for 45 sec, followed by addition of 25 ul potassium metabisulfate (0.5 mg/ml) and 10 ul BSA (10 mg/ml). Unreacted ^{125}I was separated by using a Sephadex G25 column (1.6 x 30 cm).

Endonuclease reaction buffer (0.01 M Tris-HCl pH 7.4, 0.1 M NaCl, 0.002 M magnesium chloride), 50 ug HAd5 ^{14}C-DNA and 10 ul of ^{125}I-labeled protein were incubated at 37°C for 1 min and the reaction stopped by addition of EDTA to a final concentration of 0.05 M.

The mixture was submitted to equilibrium centrifugation in 4 M guanidine HCl, 3 M CsCl, 10 mM Tris, 1 mM EDTA, pH 7.0, at 100,000 xg in a Beckman SW50 rotor for 64 h at 20°C. Following centrifugation, fractions were collected and counted for ^{14}C and ^{125}I.

12 Analytical centrifugation

5 ug HAd5 DNA and 10 ug viral protein were reacted in endonuclease assay buffer at 37°C for 1 min and submitted to analytical centrifugation in 4 M guanidine HCl, 3 M CsCl, 10 mM Tris, 1 mM EDTA, pH 7.0, at 44,000 rpm, in a Beckman model E centrifuge. The mixture was adjusted to 1.477 g/ml and the refractive index to between 1.4350 and 1.4360. After 23.5 h of centrifugation, the gradient was relaxed by reducing the centrifuge speed to 30,000 rpm for a further 29.5 h. 0.2 ml of the mixture was withdrawn at the end of the relaxation run and 0.25 ml of the guanidine HCl solution was added and the mixture centrifuged to equilibrium at 44,000 rpm for 23.0 h. Pictures were taken at different times for the examination of the DNA banding.

13 Ion-exchange chromatography

DEAE-cellulose (Sigma Chemical Company, St. Louis, Mo.)

was prepared as a 20% (w/v) suspension. 600 ml 0.5 N HCl was used to wash the suspension followed by 600 ml of 0.5 N NaOH. Washing was continued with several volumes of TMB. A 1.6 x 30 cm column was packed by gravity then washed with 50 ml of equilibrating buffer (TMB). Samples containing endonuclease activity were layered on the column and eluted with a linear gradient of 0-0.5 M NaCl in TMB. Conductivity of each fraction was measured with a conductivity salt bridge apparatus (Model 31, Yellow Springs Instruments, Yellow Springs, Ohio.) and molarity was determined by using a computer-generated 'best fit' (least squares) straight line program.

Sephadex ion-exchange matrices A25, A50 and C25 were prepared as above. Absorbance, hemagglutination enchancement, two-dimensional immuno electrophoresis, and endonuclease activity assays were performed on the eluted column fractions. Fractions showing positive endonuclease activity were pooled, concentrated by polyethylene glycol 6000 (PEG-6000, Baker Chemicals) and dialysed against TMB at room temperature overnight before layering onto an Affi-Gel Blue (BioRad Laboratories, Mississauga, Ont.) column.

The Affi-Gel Blue matrix was equilibrated with TMB and packed by gravity in a 1.6 x 15 cm column. The bed volume of

Affi-Gel Blue is dependent upon on the amount of protein added to the column (1 ml bed volume to 4 mg of protein). A dialysed sample was layered on top of the column and eluted with TMB.

14 Immunoelectrophoresis

Agarose gel immunoelectrophoresis plates (8 x 10 cm) were prepared by addition of 15 ml of melted 1% (w/v) agarose (BioRad Laboratories) in 0.02 M Veronal buffer, pH 8.6. After solidification of agar, sample wells were made by means of a 3 mm diameter punch, template and guide. Five ul of sample were added to each well, and the plate placed in a horizontal electrophoresis apparatus (BioRad Laboratories, Model 1400 Electrophoresis Cell) using "Telfa" paper wicks as the buffer-gel connection. First dimension electrophoresis was carried out at a constant voltage of 8 V/cm for 2 h with water cooling.

On completion of the first dimension electrophoresis, a gel strip containing the migrated antigen was cut out and placed on another plate. Twelve ml of 1% agarose in Veronal buffer and 100 ul of antiserum were mixed and added to the plate surrounding the gel strip. The plate was then electrophoresed in the second dimension (at right angle to

the first dimension) at 3 V/cm for 16 h. At the end of the run, the glass plate was dried and stained as described below: the gel was pressed under a layer of filter paper and a 3 cm layer of blotting paper for about 15 min. The filter paper was removed and the glass plate was dried with a hot air blower. The plate was stained with 0.2% Coomassie Brillant Blue R dissolved in acetic acid:methanol:water (7:5:88, respectively) for 5 min followed by destaining with the same solution (less stain). The plates were pressed and dried as described above.

15 SDS-polyacrylamide gel electrophoresis of virion protein

SDS-polyacrylamide slab-gels were prepared using a modification of the method described by Maizel (1971). The polyacrylamide slab-gels were prepared in a BioRad model 220 electrophoresis apparatus (150 x 100 x 1.5 mm) with a 3% stacking gel above a 13% resolving gel (acrylamide-bisacrylamide ratio of 30:0.8). After polymerization of the resolving gel (20 ml volume), a slotted comb was inserted into the apparatus, followed by addition of the stacking gel (10 ml volume). After polymerization of the gel, the comb was removed and the formed slots were filled with electrode

buffer.

Samples for analysis were prepared in the following way. Purified virus protein concentration was determined by spectrophotometric measurement and virus soluble component concentration by means of the BioRad protein assay system (Bradford, 1976). 50-100 ug of protein of each sample was drop-dialysed against 1% ammonium acetate at room temperature. Dissociation buffer was added to a final concentration of 0.05 M Tris-HCl, pH 7.6, 1% sodium dodecyl sulphate, 0.1% beta-mercaptoethanol, 10% glycerol and 0.002% bromophenol blue in the samples. Electrophoresis was carried out at a constant current of 30 mA for 220 min. At the end of the electrophoresis, the slab gel was removed, stained overnight in 0.2%. Coomassie Brilliant Blue R solution and destained as described earlier. The MW of the polypeptides revealed on each gel was determined by comparison with the relative mobility of HAd5 or HAd2 polypeptides electrophoresed in the same gel. MW calculation was done by using a computer generated "best fit" (least squares) straight line program.

16 Buffer solutions

Buffer solutions required for the course of this study

were prepared from tables listed by Gomori (1955).

17 Manuscript production

This thesis was drafted, edited and printed using the FMT program and the Amdahl 470V/6 computer of Computing Services, The University of Alberta.

RESULTS

1 Purification of AAEL-activity from virions.

HAd5 virions from three cycles of equilibrium ultracentrifugation were utilized for the isolation of a protein exhibiting AAEL-activity. The procedure involved is summarized schematically in Fig. 1. The high-speed centrifugation (80,000 xg) supernatant from disrupted virions was subjected to column chromatography

a) Affi-Gel Blue chromatography

The elution pattern from Affi-Gel Blue chromatography was monitored by absorbance readings at 280 nm (Fig. 2) Only one peak at 280 nm (designated P1) was obtained, which corresponded to the void volume. When all fractions were subjected to hemagglutination enhancement assay (HE), the peak at 280 nm showed very low titer but fractions 18-20 (P2) demonstrated high HE activity. P1 and P2 were positive for AAEL- activity as detected by spectrofluorimetric assay. P1 was further purified by Affi-Gel Blue chromatograpy (2X) and repeatedly appeared in the column void volume (Fig. 3)

Fig. 1

Purification of AAEL-activity protein from HAd5 virions.

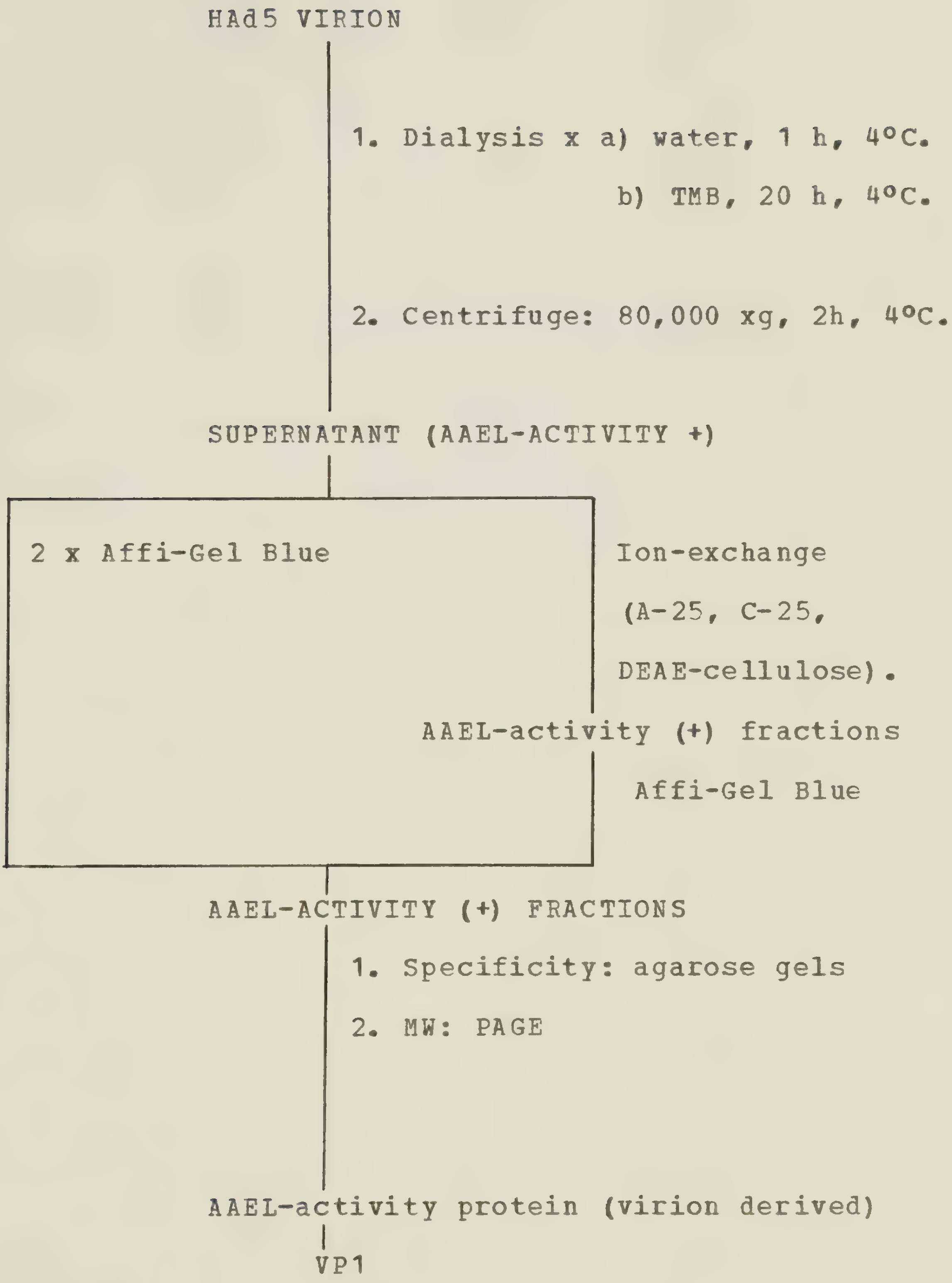

HAd5 VIRION
1. Dialysis x a) water, 1 h, 4°C.
b) TMB, 20 h, 4°C.
2. Centrifuge: 80,000 xg, 2h, 4°C.
SUPERNATANT (AAEL-ACTIVITY +)
2 x Affi-Gel Blue
Ion-exchange
(A-25, C-25,
DEAE-cellulose).
AAEL-activity (+) fractions
Affi-Gel Blue
AAEL-ACTIVITY (+) FRACTIONS
1. Specificity: agarose gels
2. MW: PAGE
AAEL-activity protein (virion derived)
VP1

Fig. 2

Purification of AAEL-activity protein from HAd5 virions by Affi-Gel Blue chromatography.

80,000 xg supernatant was obtained and chromatographed as described in Materials and methods.

(•–•): Absorbance at 280 nm

(xxx): AAEL-activity detected by spectrofluorimetric assay

(ooo): HE activity

(——): NaCl gradient

Vo : void volume

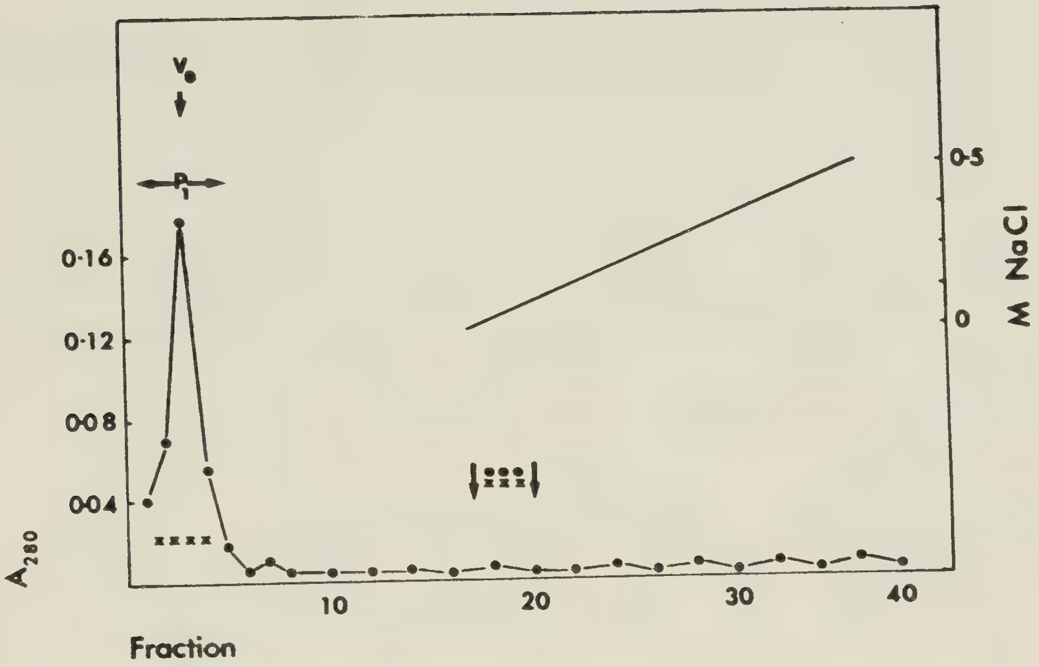

V_e
P_1
0·16
0·12
0·08
0·04
A_280
0·5
0
M NaCl
10
20
30
40
Fraction

Fig. 3

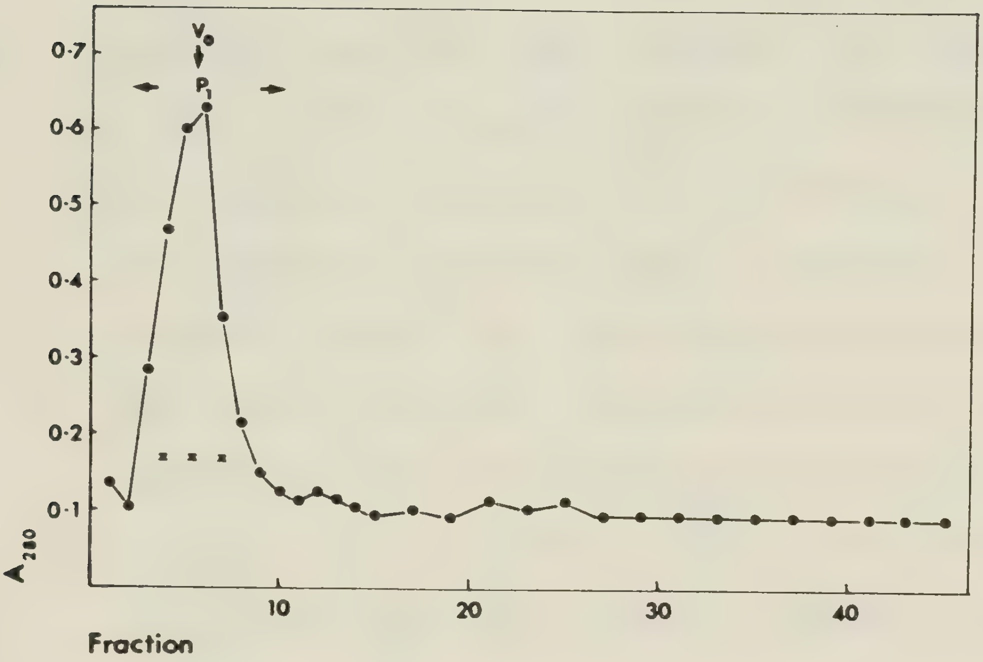
Purification of AAEL-activity protein from HAd5 by Affi-Gel Blue chromatography.

P1 from Affi-Gel Blue chromatography (Fig. 2) was pooled, concentrated, dialysed and further purified by chromatography through a second Affi-Gel blue column.

(●—●): Absorbance at 280 nm

(xxx): AAEL activity

Vo : void volume


V0
P1
0·7
0·6
0·5
0·4
0·3
0·2
0·1
A280
10
20
30
40
Fraction

b) Ion-exchange chromatography

When the 80,000 xg supernatant was subjected to cation-exchange (CM-Sephadex C25) chromatography (Fig. 4), two peaks of AAEL-activity were obtained. The first peak showed both HE and AAEL-activities, but the second peak (eluting at approximately 0.14 M NaCl) showed no HE activity. Since the first peak appeared in the void volume, an anion exchange column (DEAE-Sephadex A25) was then employed with the result being illustrated in Fig. 5.

The elution profile shows two peaks of AAEL-activity with the first having no HE activity and the second with HE activity. Results from DEAE-cellulose column chromatography showed only one peak eluting in the void volume with both types of activities (Fig. 6). When this peak was subjected to Affi-Gel Blue chromatography, a similar elution pattern was obtained (cf. Fig. 3).

2 Purification of AAEL-activity associated with soluble components.

The purification scheme is summarized in Fig. 7. Soluble components were precipitated by ammonium sulphate,

Fig. 4

Purification of HAd5-derived 80,000 xg supernatant by cation-exchange (CM-Sephadex C25) chromatography

(ooo): HE activity

(——): NaCl gradient

(•–•): Change in fluorescence units after heating, as determined by spectrofluorimetric assay using PM2 DNA as substrate

Vo : void volume

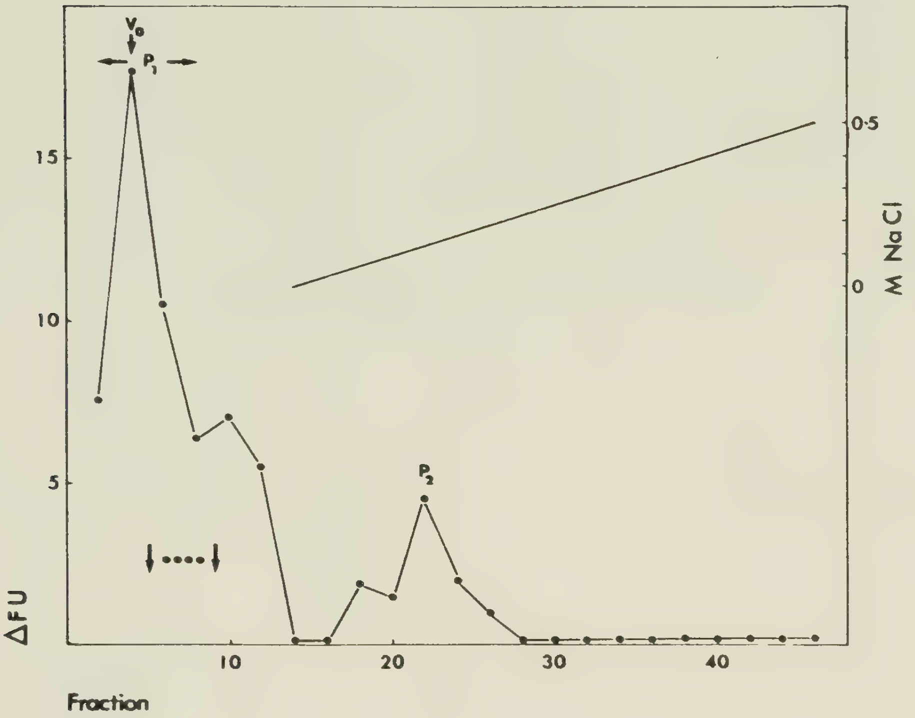

V₀
P₁
P₂
15
10
5
ΔFU
0·5
0
M NaCl
10
20
30
40
Fraction

Fig. 5

Chromatography of HAd5-derived 80,000 xg supernatant by anion-exchange (DEAE-Sephadex A25) chromatography.

(•–•): change in fluoresence units after heating as detected by spectrofluorimetric assay

(ooo): HE activity

(——): NaCl gradient

Vo : void volume

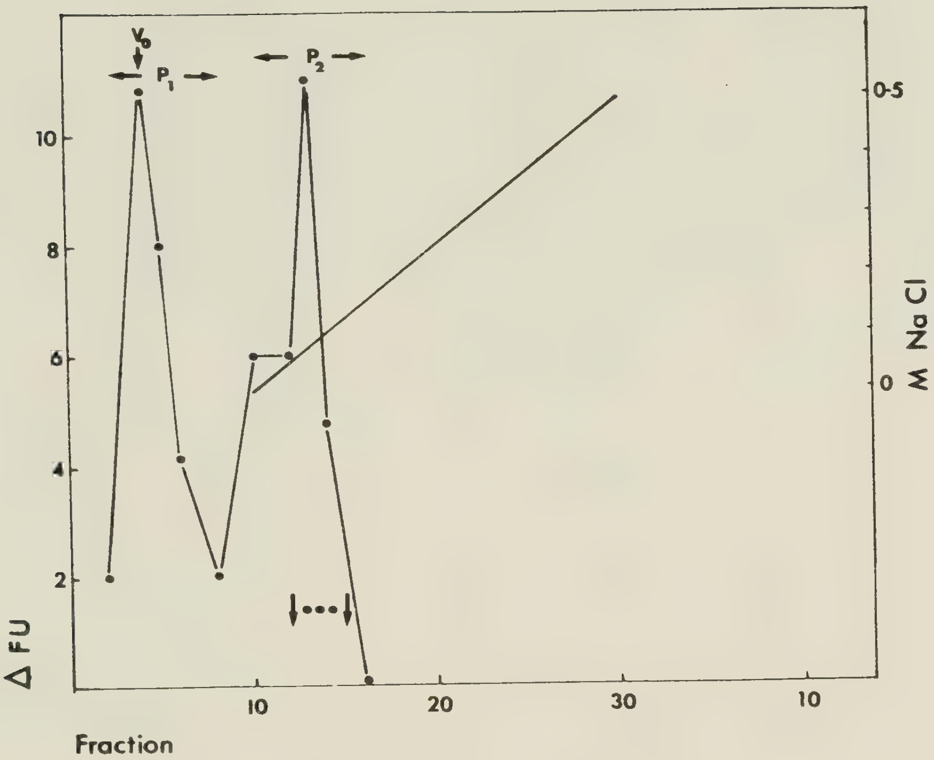

V_0
P_1
P_2
10
8
6
4
2
Δ FU
0-5
0
M Na Cl
10
20
30
10
Fraction

Fig. 6

Chromatography of HAd5-derived 80,000 xg supernatant by anion-exchange (DEAE-cellulose) chromatography.

(•–•): Change in fluorescence units after heating

(ooo): HE activity

(——): NaCl gradient

P1 eluted in the void volume

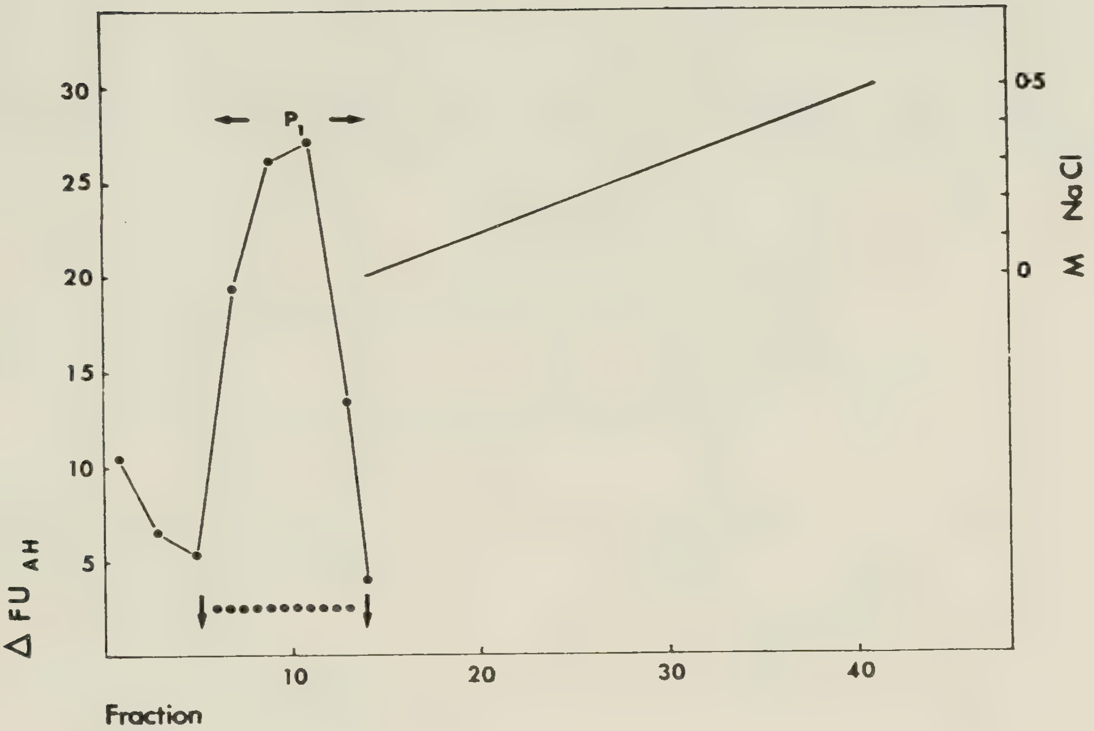
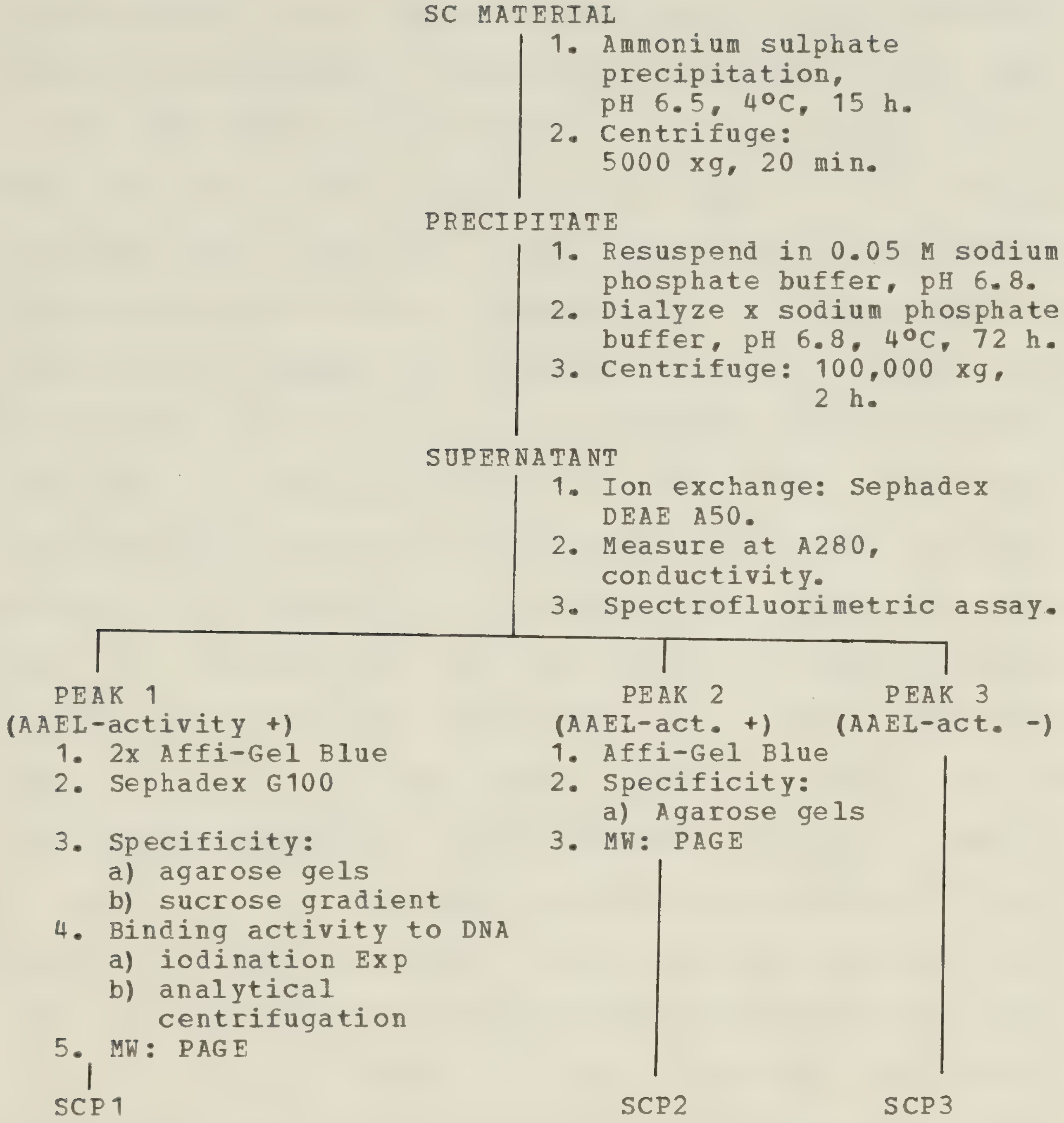

P1
30
25
20
15
10
5
Δ FU AH
10
20
30
40
Fraction
0·5
0
M NaCl

Fig. 7

Purification of AAEL-activity protein from soluble components of HAd5.



SC MATERIAL

1. Ammonium sulphate precipitation, pH 6.5, 4°C, 15 h.
2. Centrifuge: 5000 xg, 20 min.

PRECIPITATE

1. Resuspend in 0.05 M sodium phosphate buffer, pH 6.8.
2. Dialyze x sodium phosphate buffer, pH 6.8, 4°C, 72 h.
3. Centrifuge: 100,000 xg, 2 h.

SUPERNATANT

1. Ion exchange: Sephadex DEAE A50.
2. Measure at A280, conductivity.
3. Spectrofluorimetric assay.

PEAK 1
(AAEL-activity +)

1. 2x Affi-Gel Blue
2. Sephadex G100
3. Specificity:
   a) agarose gels
   b) sucrose gradient
4. Binding activity to DNA
   a) iodination Exp
   b) analytical centrifugation
5. MW: PAGE

SCP1

PEAK 2
(AAEL-act. +)

1. Affi-Gel Blue
2. Specificity:
   a) Agarose gels
3. MW: PAGE

SCP2

PEAK 3
(AAEL-act. -)

SCP3

dialyzed, centrifuged, then subjected to anion exchange (DEAE-Sephadex A50) column chromatography (Fig. 8). Three peaks with absorbance at 280 nm were obtained in which peak 1 had only AAEL-activity with very low HE activity, peak 2 had both AAEL- and HE activities and peak 3 had neither activity. When fractions from peaks 1 and 2 were further purified by Affi-Gel Blue chromatography, the first peak of AAEL-activity eluted in the void volume,(Fig. 9, 10). The second peak activity also showed a similar elution pattern but with a decrease in HE titer (Fig. 11). In exclusion chromatography (Sephadex G100) the first peak of AAEL-activity again appeared in the column void volume (Fig. 12). From the results of the above described chromatographic procedures, the protein exhibiting AAEL-activity did not bind to any of the column materials used, but as purification progressed, impurities were, in fact, removed as shown by the decrease in number of polypeptide bands appearing in PAGE gels (Fig. 13, 14) and the appearance of only one peak in two-dimensional immunoelectrophoresis (Fig. 15). As shown in Table 1, there was also a significant increase in specific activity during each progressive step.

Fig. 8

Purification of AAEL-activity protein from HAd5 SC material by anion-exchange (DEAE-Sephadex A50) chromatography.

SC material prepared as described in Materials and Methods, was chromatographed on DEAE-Sephadex A50.

(•—•): Absorbance at 280 nm

(xxx): AAEL-activity

(o-o): HE activity

(——): NaCl gradient

Vo : void volume.

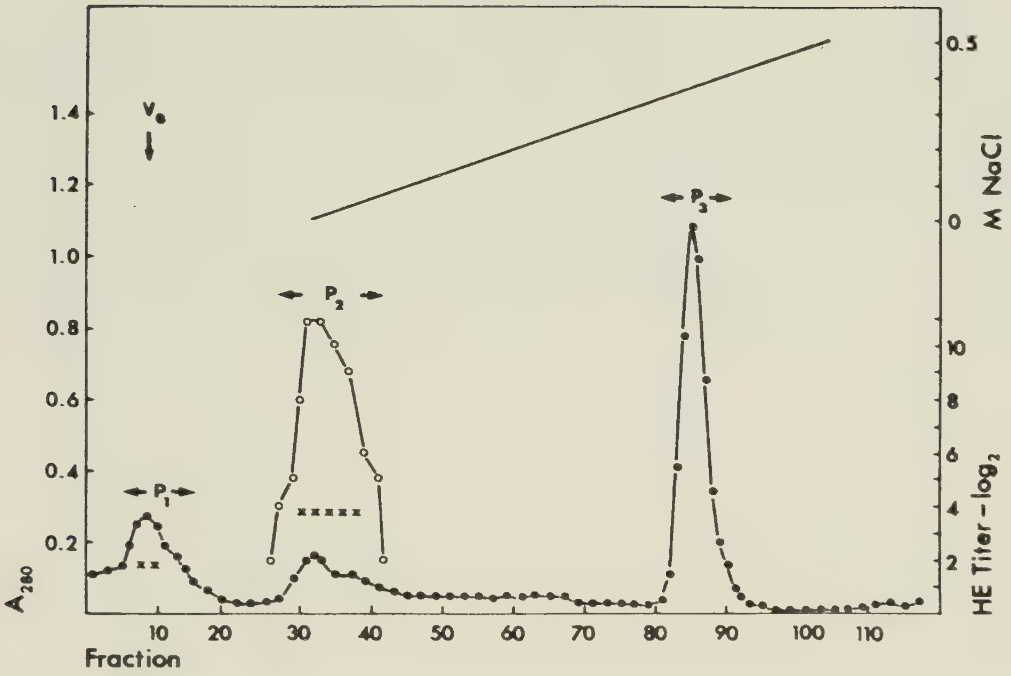

1.4
1.2
1.0
0.8
0.6
0.4
0.2
A280
V0
P1
P2
P3
0.5
0
M NaCl
10
8
6
4
2
HE Titer – log2
10
20
30
40
50
60
70
80
90
100
110
Fraction

Fig. 9

Purification of HAd5 SC-derived AAEL-activity protein by Affi-Gel Blue chromatography.

SCP1 from DEAE-Sephadex A50 chromatography (Fig. 8) was concentrated, dialysed against TMB and eluted from an Affi-Gel Blue column with TMB.

(•—•): Absorbance at 280 nm

(xxx): AAEL-activity

Vo : void volume

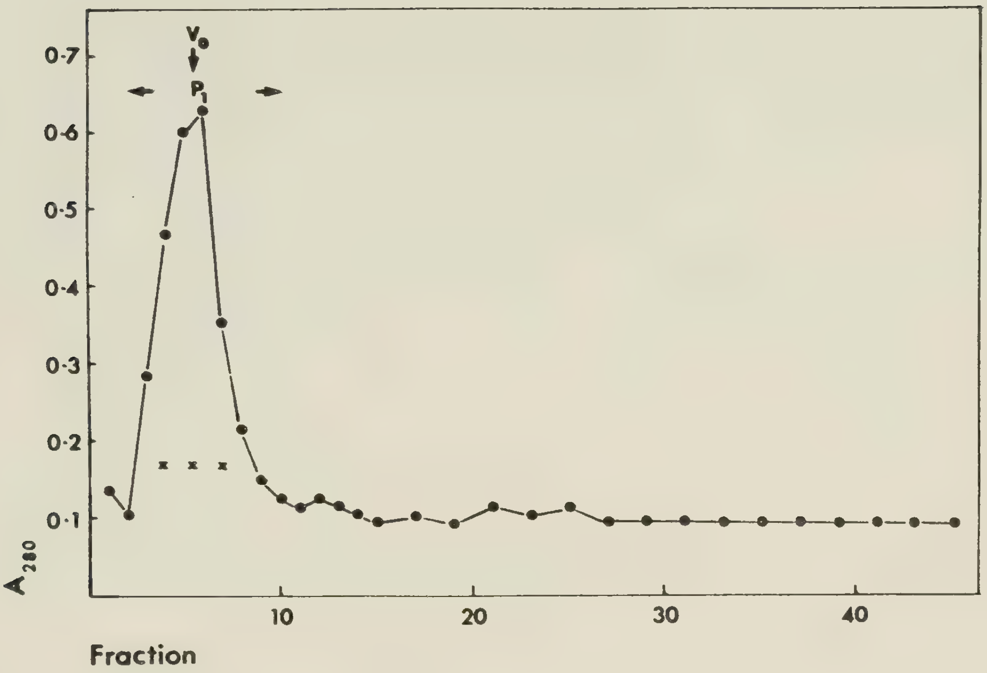

V_0
P_1
0·7
0·6
0·5
0·4
0·3
0·2
0·1
A_{280}
10
20
30
40
Fraction

Fig. 10

Purification of HAd5 SC-derived AAEL-activity protein.

SCP1 from Fig. 8 and 9 was further purified by Affi-Gel Blue chromatography.

(•—•): Absorbance at 280 nm
(xxx): AAEL-activity
Vo : void volume

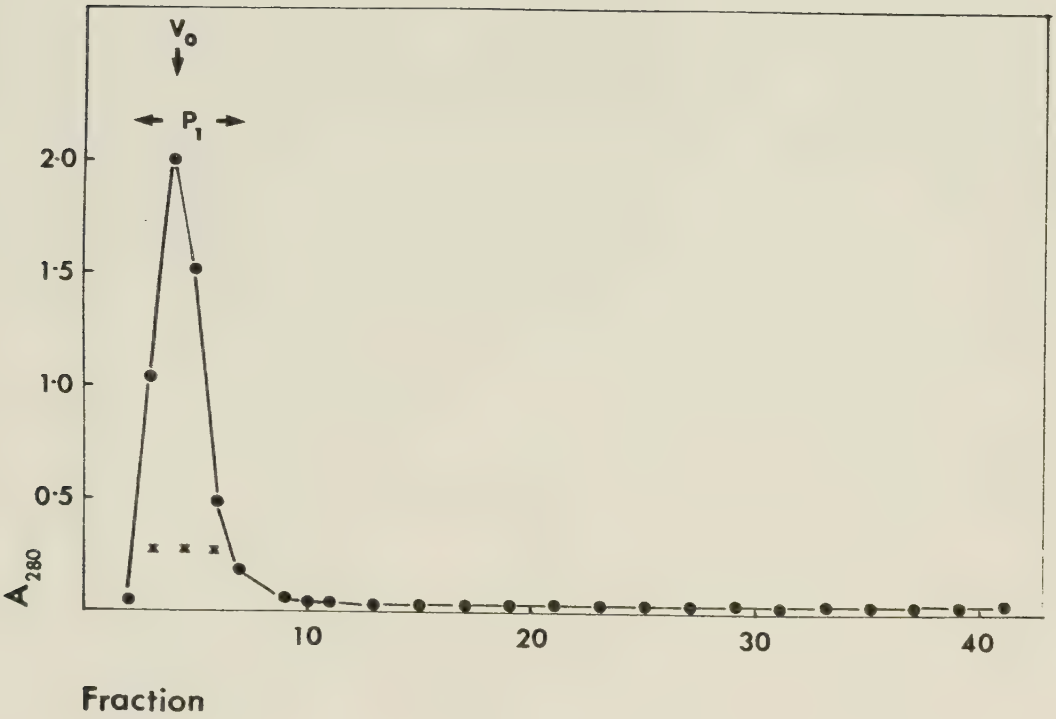

V₀
P₁
2·0
1·5
1·0
0·5
A₂₈₀
10
20
30
40
Fraction

Fig. 11

Purification of HAd5 SC-derived AAEL-activity protein by Affi-Gel Blue chromatography.

SCP2 from Fig. 8 was further purified by Affi-Gel Blue chromatography.

(●—●): Absorbance at 280 nm

(xxx): AAEL-activity

Vo : void volume

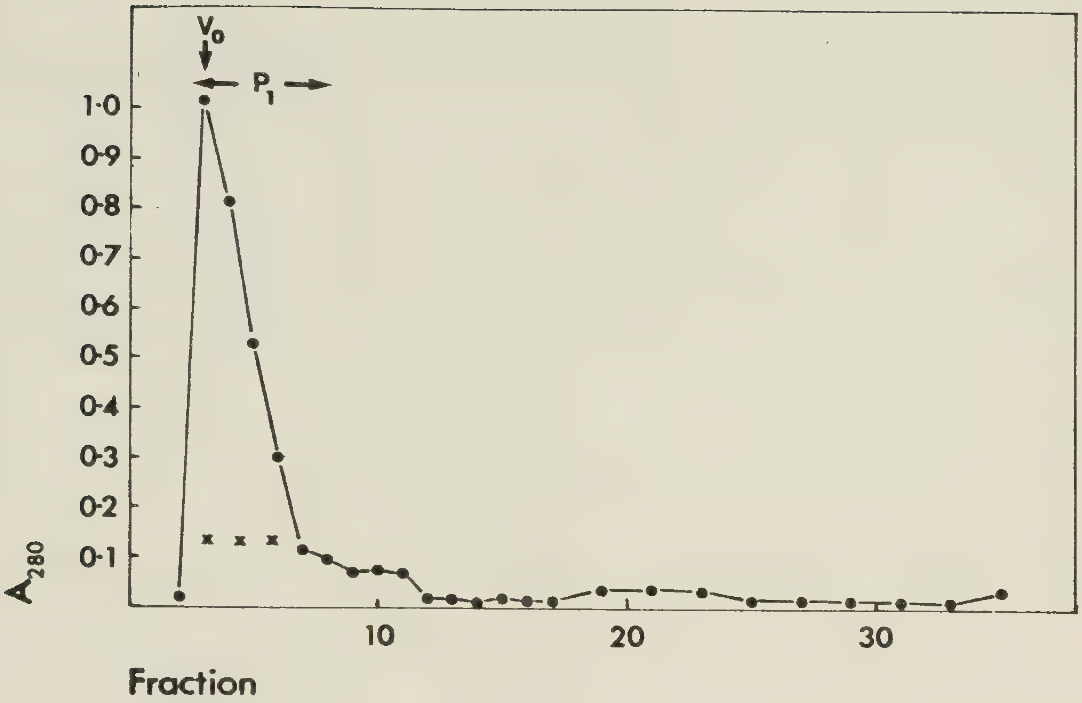

V₀
P₁
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
A₂₈₀
10
20
30
Fraction

Fig. 12

Purification of HAd5 SC-derived AAEL-activity by gel exclusion (G100) chromatography.

SCP from Fig. 10 was further purified by Sephadex G100 chromatography as described in Materials and Methods.

(●—●): Absorbance at 280 nm

(xxx): AAEL-activity

Vo : void volume

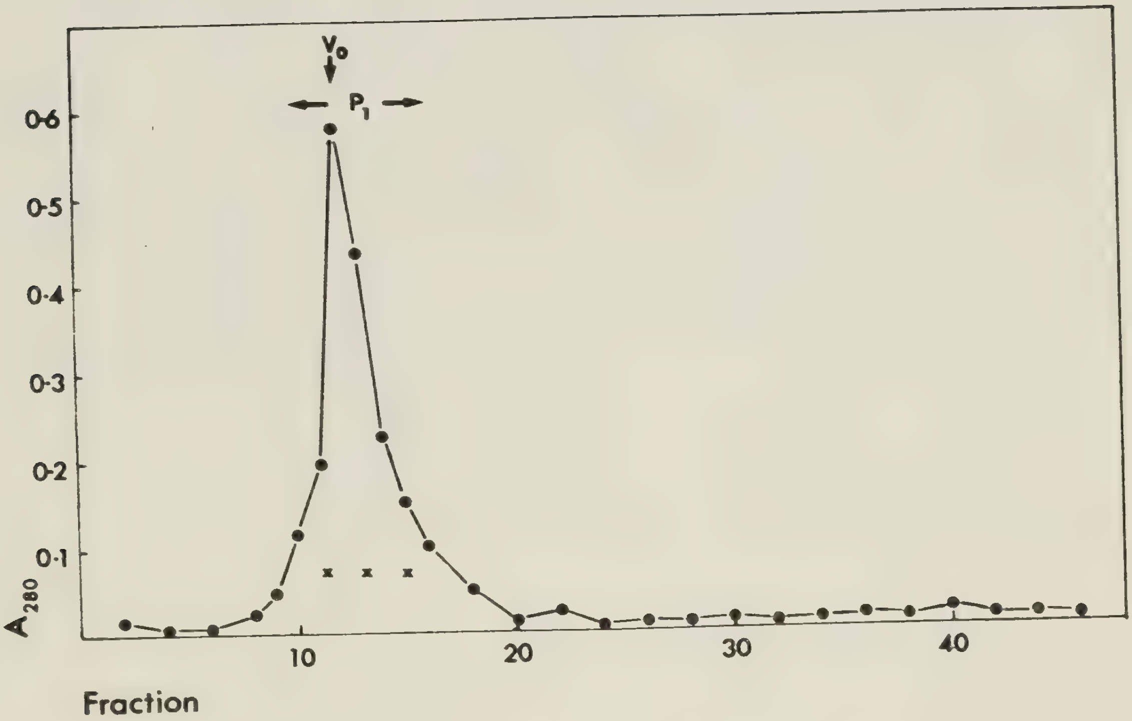

V₀
P₁
0·6
0·5
0·4
0·3
0·2
0·1
A₂₈₀
10
20
30
40
Fraction

Fig. 13

PAGE analysis of purification of AAEL-activity protein from HAd5.

Samples were prepared (as described in Materials and Methods) and electrophoresed at 30 mA constant current for 220 min.

1: HAd 5

2: SC from HAd5

3: VP1 from 2x Affi-Gel Blue chromatography (Fig. 3)

4: VP2 from 1x Affi-Gel Blue chromatography (Fig. 2)

5: HAd5

*: AAEL-activity protein

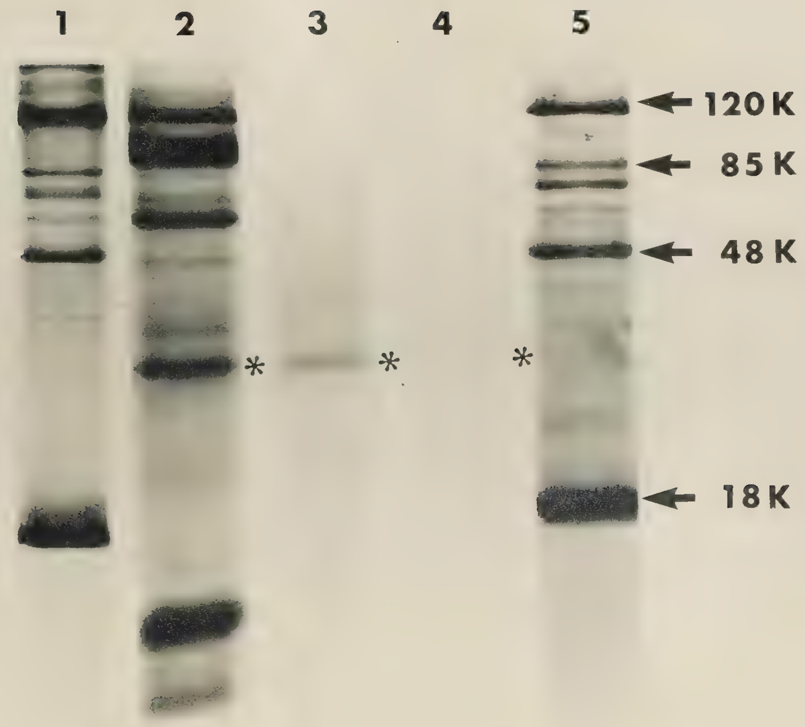

1
2
3
4
5
*
*
*
120K
85K
48K
18K

Fig. 14

PAGE analysis of the purification of AAEL-activity protein from HAd5 SC.

Samples were prepared as described in Materials and Methods.

1: HAd5

2: HAd5 SC

3: SCP1 from anion-exchange chromatography (DEAE-Sephadex A50, Fig. 8)

4: same as 3

5: SCP1 from 2x Affi-Gel Blue, G100 chromatography (Fig. 12)

6: SCP2 from 1x Affi-Gel Blue (Fig. 11)

7: SCP2 from anion-exchange chromatography (Fig. 8)

8: HAd5

*: AAEL-activity protein

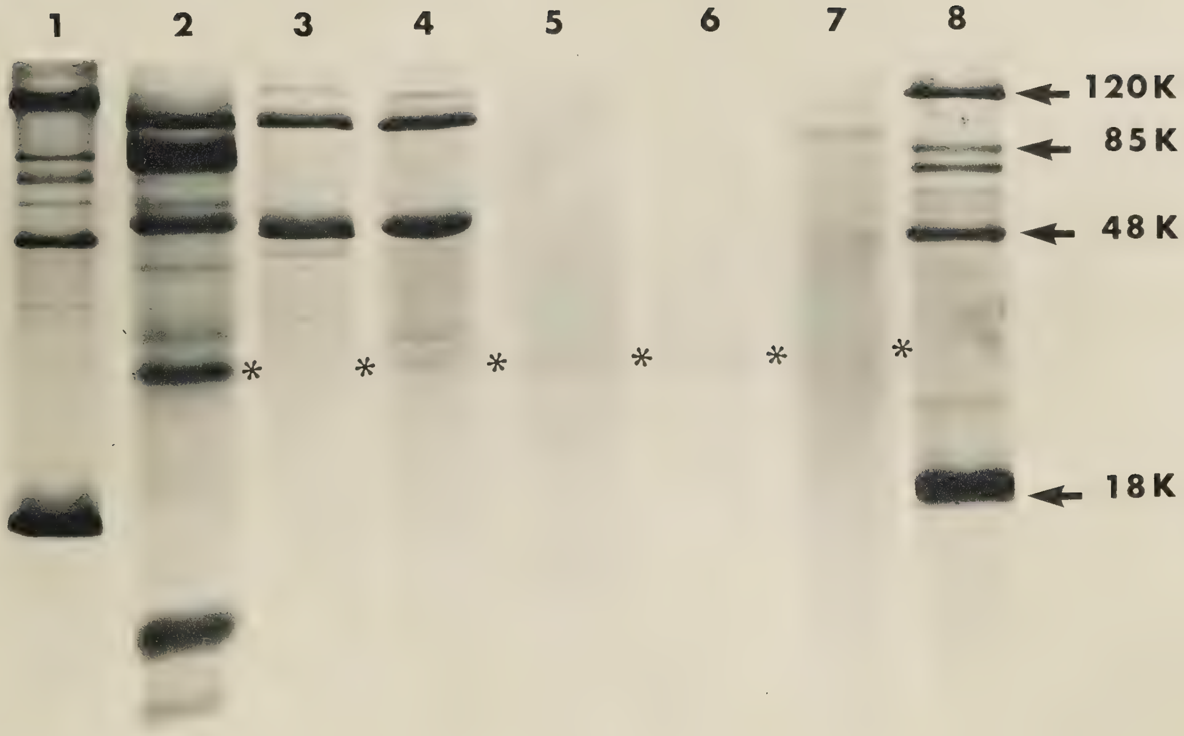

1
2
3
4
5
6
7
8
120K
85K
48K
18K
*
*
*
*
*
*

Fig. 15

Two-dimensional immunoelectrophoresis of HAd5 SC material and AAEL-activity protein from Sephadex G100 exclusion chromatography.

SC material was electrophoresed in the first dimension for 2 h at 8 V/cm and in the second dimension (in the presence of HAd5 virion antiserum) for 16 h at 3 V/cm. AAEL-activity protein derived from HAd5 SC (SCP1) was purified from anion-exchange chromatography, 2x Affi-Gel Blue, and Sephadex G100 chromatography. Agarose gels were prepared as described in Materials and Methods.

1: Hexon

2: Penton base

3: Penton

4: Fiber

↓ SCP1

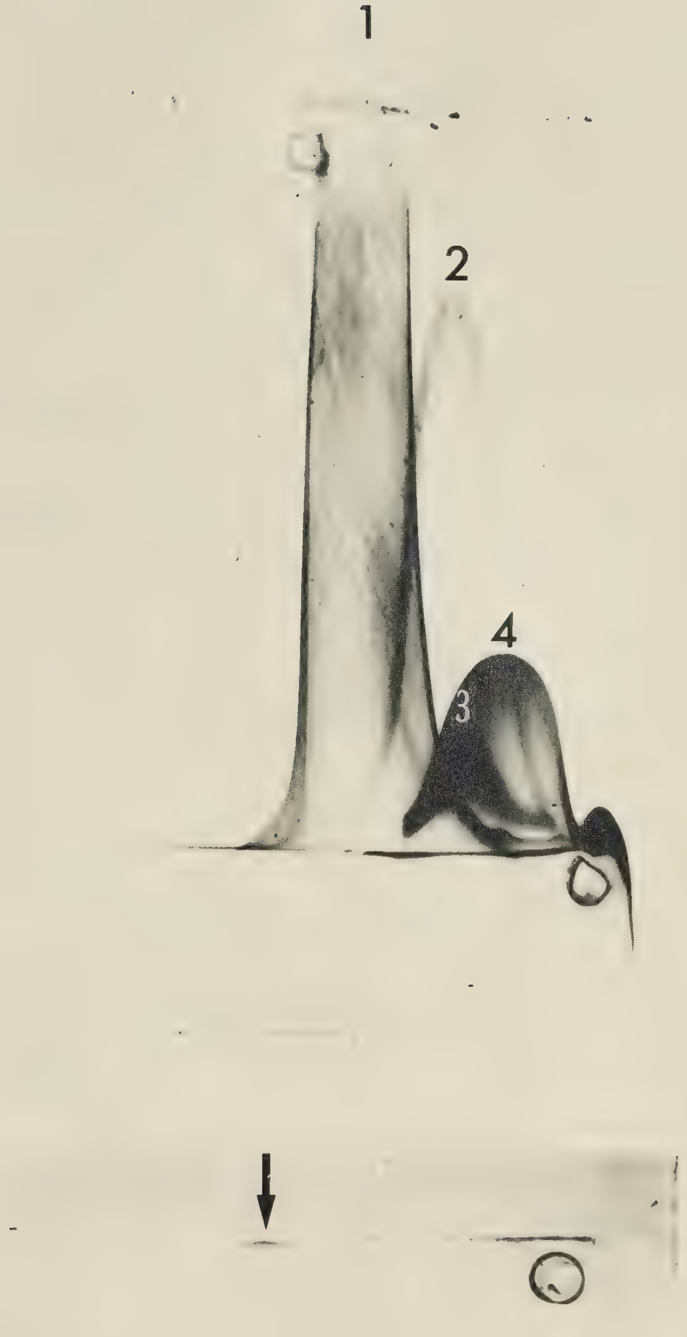


Table 1

Comparision of AAEL-activity obtained during the purification process from HAd5 SC and HAd5 disrupted virions.

$$\text{Specific activity} = \frac{\Delta \text{FU}}{\text{protein content (ug)}}$$

	FU	PROTEIN CONTENT (ug)	SPECIFIC ACTIVITY
SC			
P1 DEAE Sephadex A50	31.5	15.0	2.1
P1 2x Affi gel	38.5	9.0	4.3
P1 2x Affi gel G100	36.5	4.9	7.6
HAd5			
80,000 xg supernatant	16.0	4.6	35
P1 1x Affi gel	32.0	2.85	112
P1 2x Affi gel	18.0	1.50	120

3 Characterization of AAEL-activity protein.

a) Stability

In the 80,000 xg supernatant, the initial enzymatic activity detected via spectrofluorimetric assay (Tris buffer, pH 7.4) was minimal. After 96 h storage at room temperature, this activity significantly increased (Fig. 16). Storage of this protein (after the initial 96 h incubation) at -20°C and 4°C did not seem to further enhance this activity (Fig. 17, 18).

b) pH

When 0.01 M citrate buffer, pH 4.5, was utilized as the incubation buffer in place of the 0.01 M Tris, pH 7.4, buffer, a high amount of activity was detected as early as 24 h after storage at room temperature (Fig. 19) but this increase in activity was comparable to the activity increase after 96 h storage at room temperature in Tris buffer, pH 7.4 (Fig. 20).

c) Sensitivity to Pronase treatment

80,000 xg supernatant from disrupted HAd5 was not sensitive to Pronase treatment at a final

Fig. 16

Spectrofluorimetric assay of HAd5-derived 80,000 xg supernatant using PM2 DNA as substrate after 24 h and 96 h storage at room temperature.

FU: fluorescence unit
o : 24 h storage at room temperature
△ : 96 h storage at room temperature
—: Before heating assay mixture
--: After heating assay mixture
Tris buffer, pH 7.4, was used as incubation buffer.

Fig. 17

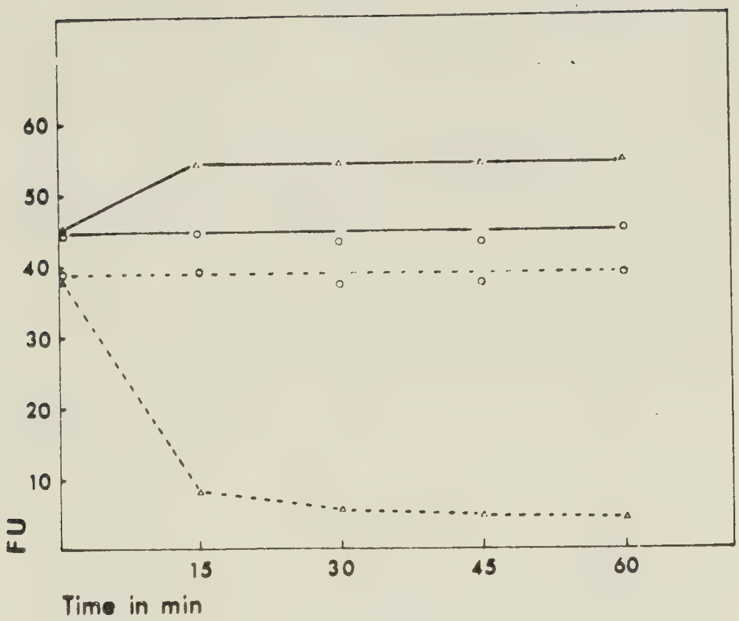
Spectrofluorimetric assay of HAd5-derived 80,000 xg supernatant after 24 h and 96 h storage at 4°C using Tris buffer, pH 7.4, as incubation buffer.

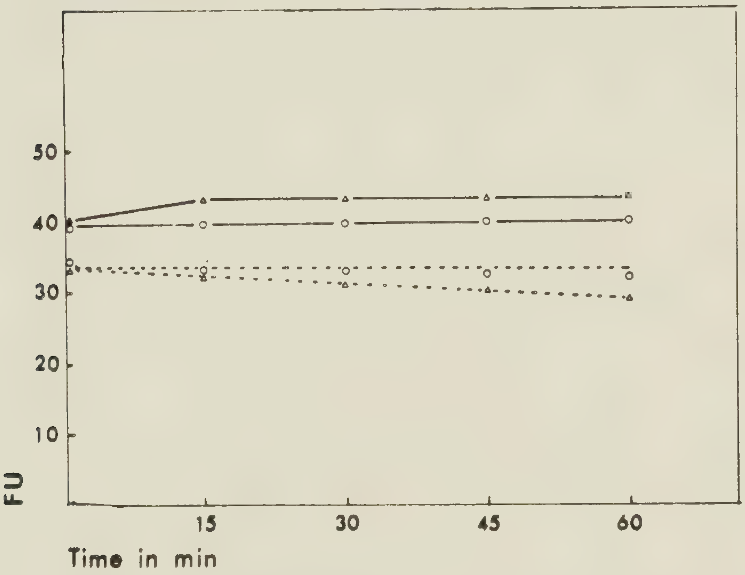
o : 24 h storage storage at 4°C
△ : 96 h storage storage at 4°C
— : Before heating assay mixture
-- : After heating assay mixture

Fig. 18

Spectrofluorimetric assay of HAd5-derived 80,000 xg supernatant after 24 h and 96 h storage at -20°C using Tris buffer, pH 7.4, as incubation buffer.

FU : fluorescence units
o : 24 h storage at -20°C
△ : 96 h storage at -20°C
— : Before heating assay mixture
-- : After heating assay mixture


60
50
40
30
20
10
FU
15
30
45
60
Time in min


50
40
30
20
10
FU
15
30
45
60
Time in min

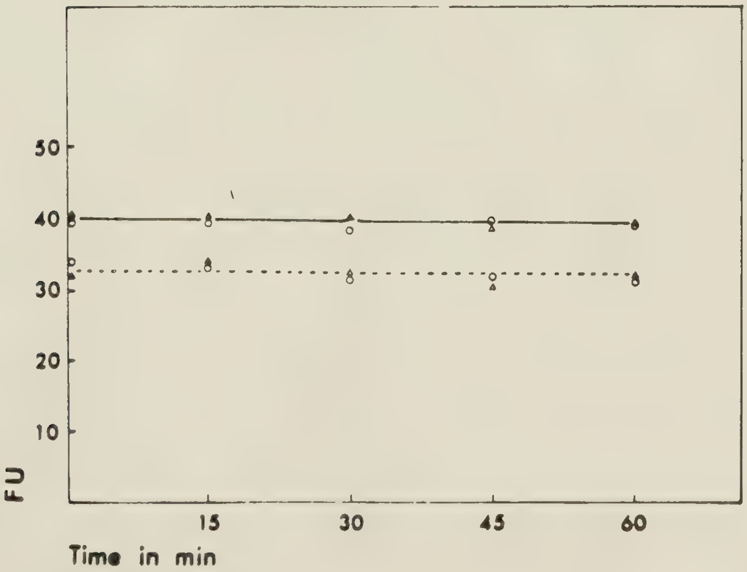

50
40
30
20
10
FU
15
30
45
60
Time in min

Fig. 19

Spectrofluorimetric assay of HAd5-derived 80,000 xg supernatant using different pH buffers in the incubation mixture after 24 h storage at room temperature.

FU : fluorescence units

▲ : 0.01 M citrate buffer, pH 4.5

o : 0.01 M Tris buffer, pH 7.4

— : Before heating assay mixture

-- : After heating assay mixture

Fig. 20

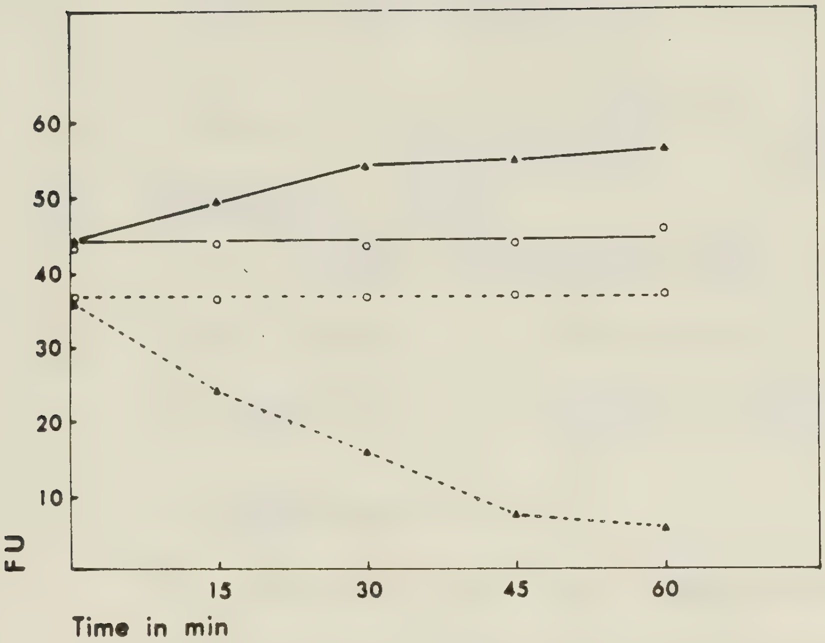
Spectrofluorimetric assay of HAd5-derived 80,000 xg supernatant using different pH buffers in the incubation mixture after 96 h storage at room temperature.

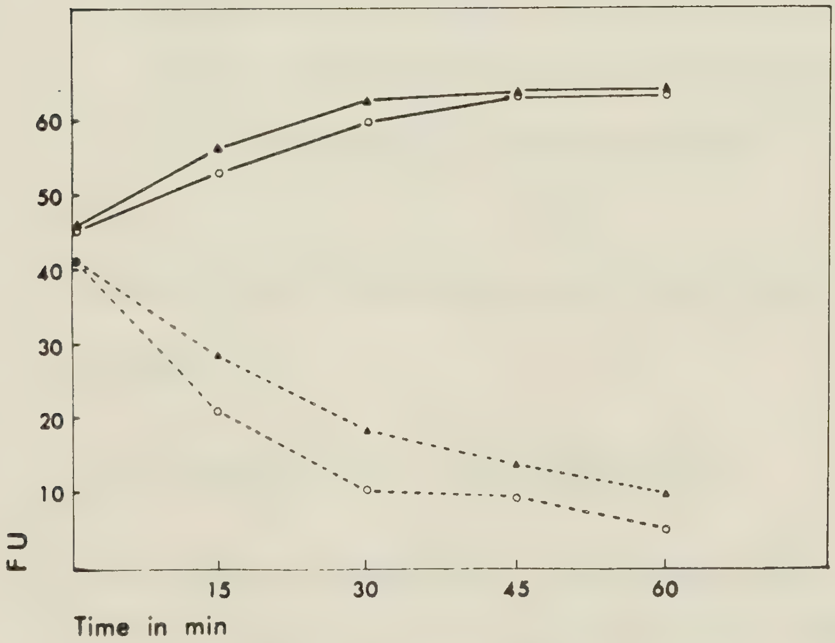
▲ : 0.01 M citrate buffer, pH 4.5

o : 0.01 M Tris buffer, pH 7.4

— : Before heating assay mixture

-- : After heating assay mixture


60
50
40
30
20
10
FU
15
30
45
60
Time in min


60
50
40
30
20
10
FU
15
30
45
60
Time in min

concentration of 200 ug/ml up to 2.5 h incubation. The activity decreased if the preparation was incubated with Pronase for 24 h at 37°C. This may be due to digestion of the protein responsible for the AAEL-activity (Table 2).

d) Effect of ion concentration, ionic detergent, and chelating agents.

The results are summarizied in Table 3. 0.5 M NaCl had no effect on AAEL-activity, whereas both EDTA (10 mM) and 5% SDS were inhibitory.

e) MW determination:

As judged from the comparative migration rate of purified protein preparations in PAGE SDS slab gels (Fig. 13, 14), the molecular weight of AAEL-activity was determined to be between 32,000 to 32,500.

4 Study of AAEL-activity site specificity and cleavage products.

I) Agarose gel electrophoresis

a) Virion-associated AAEL-activity

VP1 (Fig. 2) with AAEL-activity and VP2 (Fig. 2) with both HE and AAEL-activities, purified by gel

Table 2

Effect of Pronase treatment on HAd5-derived 80,000 xg supernatant.

Pronase at 200 ug/ml was reacted for different times with the 80,000 xg supernatant and AAEL-activity was detected by spectrofluorimetric assay.

INCUBATION TIME	CHANGE IN FU
5 min	16.5
15	14.0
30	11.5
90	12.0
2.5 h	9.5
24	4.0
control (no Pronase)	13.5

Table 3

Effects of salt, chelating agent and non-ionic detergent on AAEL-activity protein.

SCP1 from Sephadex G100 chromatography (Fig. 12) was incubated with various chemicals for 30 min and tested for AAEL-activity by spectrofluorimetric assay.

- : Presence of AAEL-activity

+ : Absence of AAEL-activity

nd: not done

CHEMICALS

	NaCl	$MgCl_2$	EDTA
1000 mM	+	nd	nd
500	-	nd	nd
200	-	nd	nd
100	nd	nd	+
50	nd	nd	+
20	nd	nd	+
10	nd	-	+
5	nd	-	nd
2	nd	-	nd
1	nd	-	nd

SDS (%)	AAEL ACTIVITY
5	+
0.5	-
0.05	-
0.005	-

chromatography, were used for this part of the study. PM2 DNA, a supercoiled plasmid DNA isolated from Pseudomonas BAL 31, was cleaved and two bands were visualized in 1.4% agarose gels (Fig. 21). Spectrofluorimetric assay showed that the 80,000 xg supernatant, VP1 from 1 x Affi-Gel Blue, and VP1 from 2 x Affi-Gel Blue were all positive for AAEL-activity. However, when the cleavage product was electrophoresed with control DNA in 1.4% agarose gels containing ethidium bromide, a very faint lower band appeared. It was postulated that not enough AAEL-activity protein had been added for the assay system. Therefore, in the following assay an increasing amount of VP1 was added to a constant amount of PM2 DNA. There was an increase in activity detected by spectrofluorimetric assay, and the intensity of the lower band also increased in proportion to the amount of protein added (Fig. 22). This result further confirmed the initial observation (Fig. 21) that there was an excess of PM2 DNA or not enough reactant to cleave the substrate.

VP1 did not seem to cleave homologous DNA, as illustrated in Fig. 23. But VP2, from Affi-Gel Blue

Fig. 21

Agarose gel electrophoresis:

Effect of cleavage by VP1 on substrate DNA throughout purification procedures.

Materials for electrophoresis were layered into wells of the slab gel apparatus. Gels were prepared as described in Materials and Methods. Electrophoresis was performed at 150 V constant voltage, for 90 min and the DNA bands were visualized under UV light.

1: Control with no VP1

2: 80,000 xg supernatant

3: VP1 from 1x Affi-Gel Blue (Fig. 2)

4: VP1 from 2x Affi-Gel Blue (Fig. 3).

5: VP1 from 2x Affi-Gel Blue (Fig. 3)

PM2 DNA was used as substrate DNA and Tris buffer, pH 7.4, as the reaction buffer.

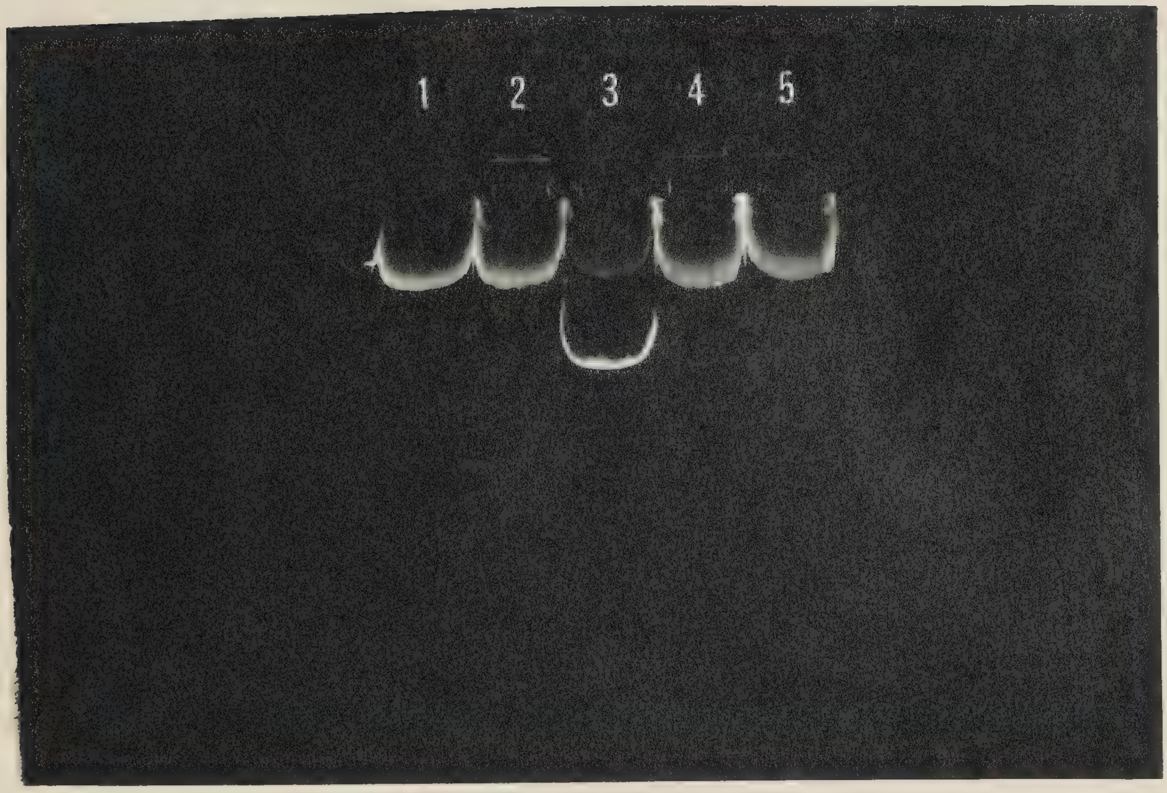

1
2
3
4
5

Fig. 22

Agarose gel electrophoresis:

Effect of AAEL-activity protein concentration on the cleavage pattern of PM2 DNA.

VP1 (Fig. 3) was used as the reactant for this assay. Details are described in Materials and Methods.

1: Control with no AAEL-activity protein

2: 10 ul of VP1

3: 20 ul of VP1

4: 30 ul of VP1

5 ug of PM2 DNA and 0.01 M Tris buffer, pH 7.4, were utilized.

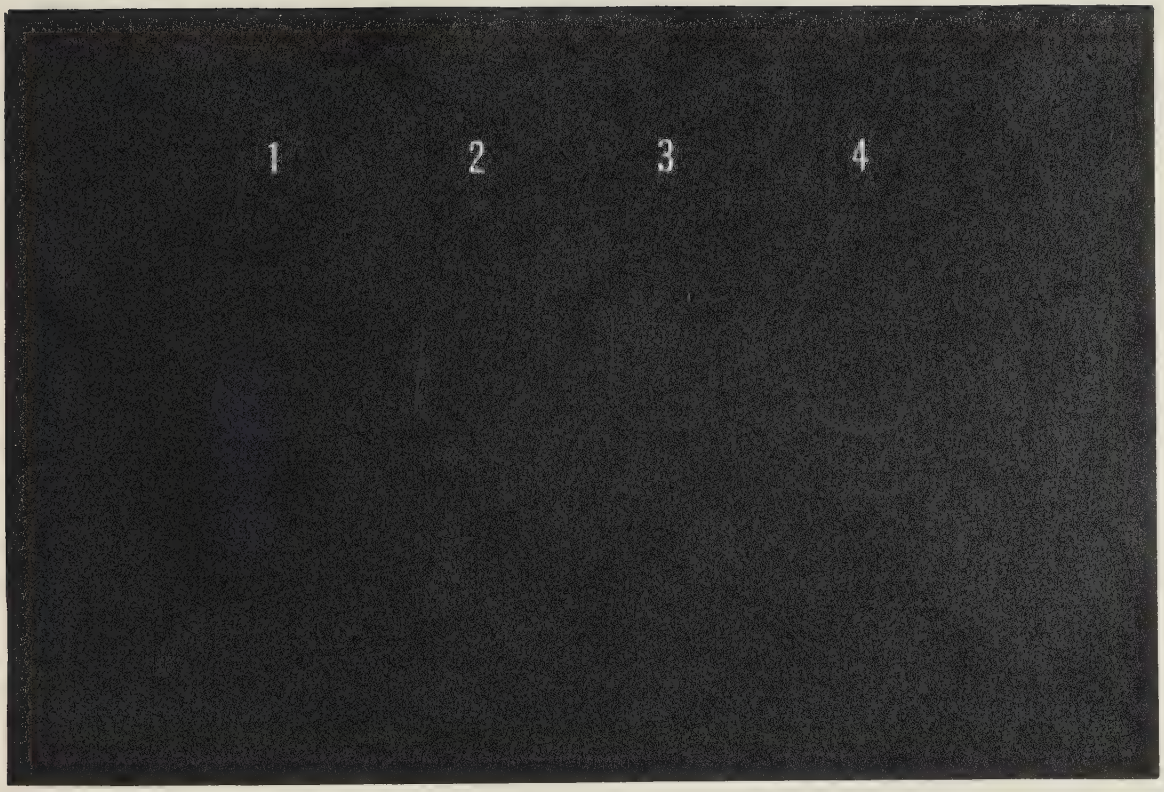

1
2
3
4

Fig. 23

Agarose gel electrophoresis:

Cleavage pattern of HAd5 DNA by VP1 (Fig. 3) and VP2 (Fig. 2) using different pH buffers.

1: control with no AAEL-activity protein

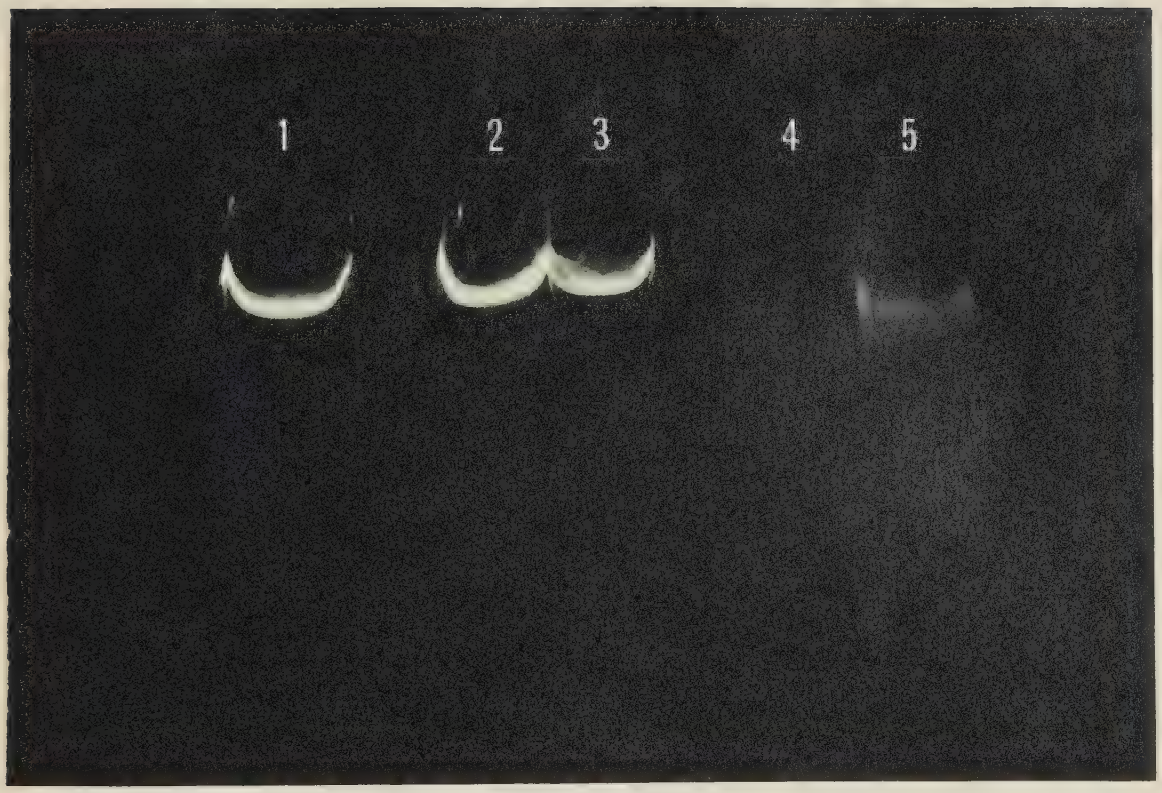
2: VP1

3: VP1

4: VP2

5: VP2

Tris buffer, pH 7.4, was used for samples 2 and 4, and citrate buffer, pH 4.5, for 1, 3 and 5. HAd5 DNA was utilized as substrate DNA.


1
2
3
4
5

chromatography, cleaved homologous DNA into various size population resulting in a smearing effect in the agarose gel. Products obtained using different pH buffers in the reaction mixture were dissimilar. In Tris buffer, pH 7.4, all the HAd5 DNA was cleaved into small fragments which migrated to the bottom of the gel (not shown in the illustrated gel). In citrate buffer, pH 4.5, (equivalent incubation period) fragmentation occured, but the cleavage product had a slower migration rate (larger size) and could still be visualized in the gel.

As no obvious cleavage was observed, (Fig. 23) in the homologous system, three times the original volume of VP1 was added to the same amount of HAd5 DNA. Fig. 24 illustrates that cleavage did occur, with a slight smearing of the DNA band as compared to the control DNA. S1 restriction enzyme, specific for cleavage of single stranded DNA opposite the primary cleavage site, was utilized but the type of banding obtained in agarose gels indicated that it did not affect the type of cleavage involved (Fig. 24).

Eco RI restriction enzyme is known to cleave HAd5 DNA into 3 distinct fragments and HAd2 DNA into 6

Fig. 24

Agarose gel electrophoresis:

Effect of cleavage of HAd5 DNA by VP1 (Fig. 3) followed by S1 restriction enzyme cleavage.

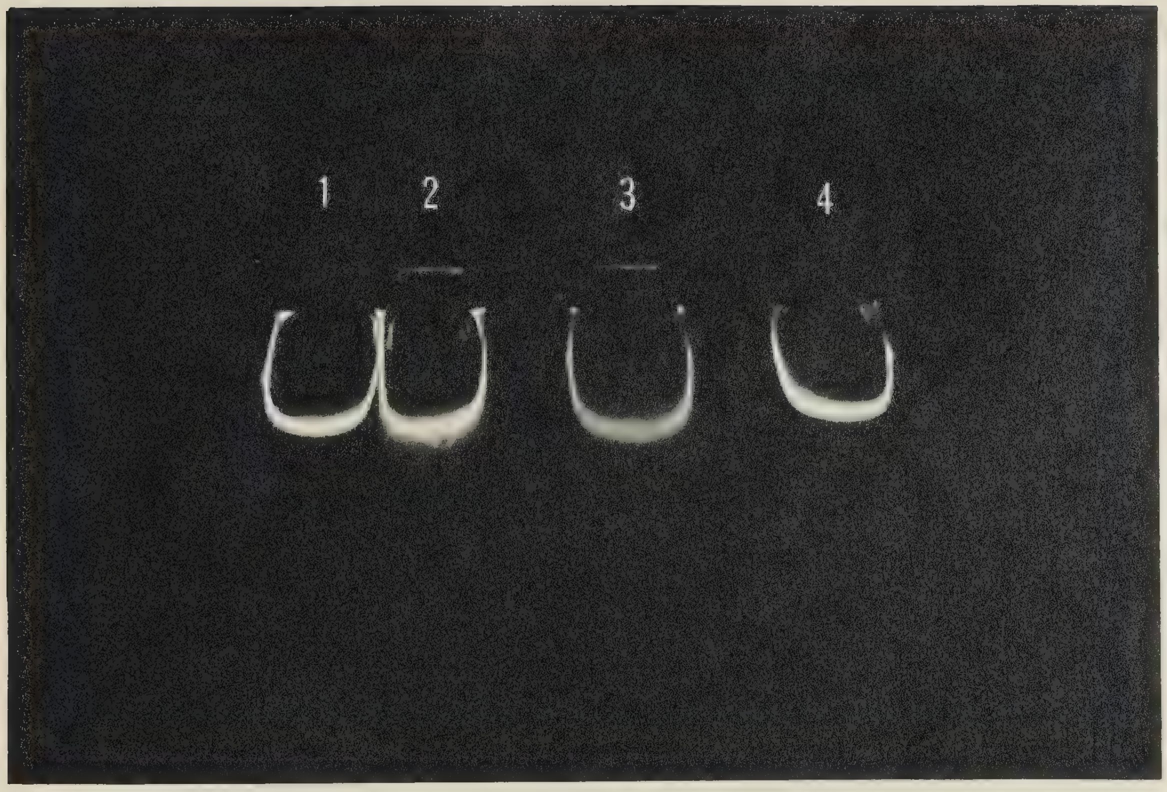
1: Control with no AAEL-activity protein

2: 30 ul VP1

3: 30 ul VP1 and S1

4: S1

PM2 DNA and Tris buffer, pH 7.4, were used.


1
2
3
4

fragments. Eco RI enzyme was utilized to cleave HAd5 DNA before or after treatment with purified VP1 (Fig. 3) from gel chromatography and no observable changes were obtained in the gels (Fig. 25)

b) SC-associated AAEL-activity protein

AAEL-activity protein SCP1 and SCP2, purified from SC material, were used in the study of site specificity. With PM2 DNA, 2 bands, with smearing of the lower band, were obtained from both SCP1 and SCP2 cleavage (Fig. 26). Using homologous HAd5 DNA as substrate, smearing of the DNA band was obtained with SCP1 or SCP2 only when the reaction mixture included Tris buffer, pH 7.4, but not with citrate buffer, pH 4.5. SCP1 and SCP2 cleaved Eco RI fragments of HAd5 DNA into a population of different sizes whether the protein were added before or after Eco RI cleavage. (Fig. 27).

II) Sucrose gradient analysis of AAEL-activity

^{14}C-HAd5 DNA was cleaved by purified SCP1 protein (2x Affi-Gel Blue, G100 column) and layered on to a 0-30% sucrose gradient above a 60% sucrose cushion, and centrifuged at 23,000 rpm for 17 h. The result obtained is illustrated in Fig. 28.

Fig. 25

Agarose gel electrophoresis:

Cleavage pattern of VP1 (Fig. 12) on HAd5 DNA before and after Eco RI restriction enzyme cleavage.

1: Control with no enzyme or AAEL-activity protein.

2: Eco RI

3: Eco RI followed by VP1

4: VP1 followed by Eco RI

5: Eco RI

6: Eco RI followed by VP1

7: VP1 followed by Eco RI

Wells 1-4 : HAd5 DNA as substrate DNA

Wells 5-7 : HAd2 DNA as substrate DNA

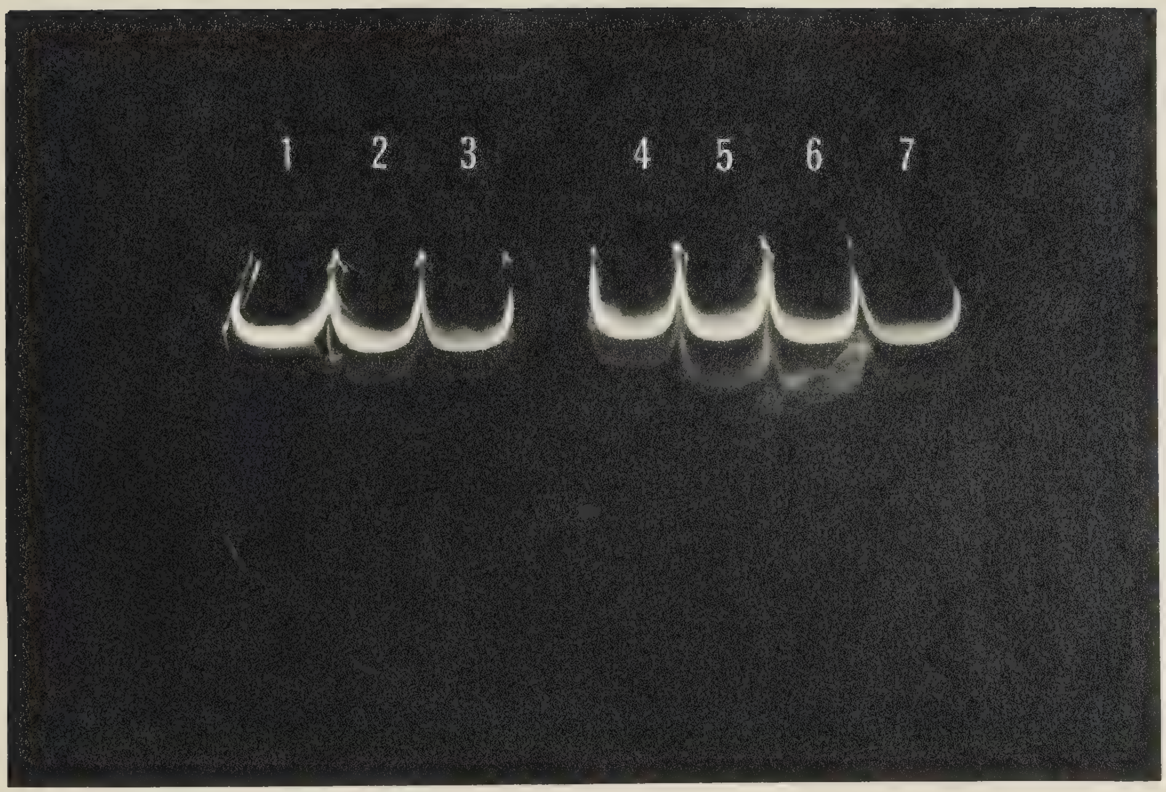

1
2
3
4
5
6
7

Fig. 26

Agarose gel electrophoresis:

Cleavage pattern of PM2 and HAd5 DNA following incubation with SCP1 (Fig. 12) and SCP2 (Fig. 11).

1: Control PM

2: SCP1

3: SCP2

4: SCP1

5: SCP1

6: SCP2

7: SCP2

8: Control

Wells 1-3 : PM2 DNA as substrate

Wells 4-8 : HAd5 DNA as substrate

Wells 1-3,4 & 6 : Tris buffer, pH 7.4

Wells 5, 7, 8 : citrate buffer, pH 4.5

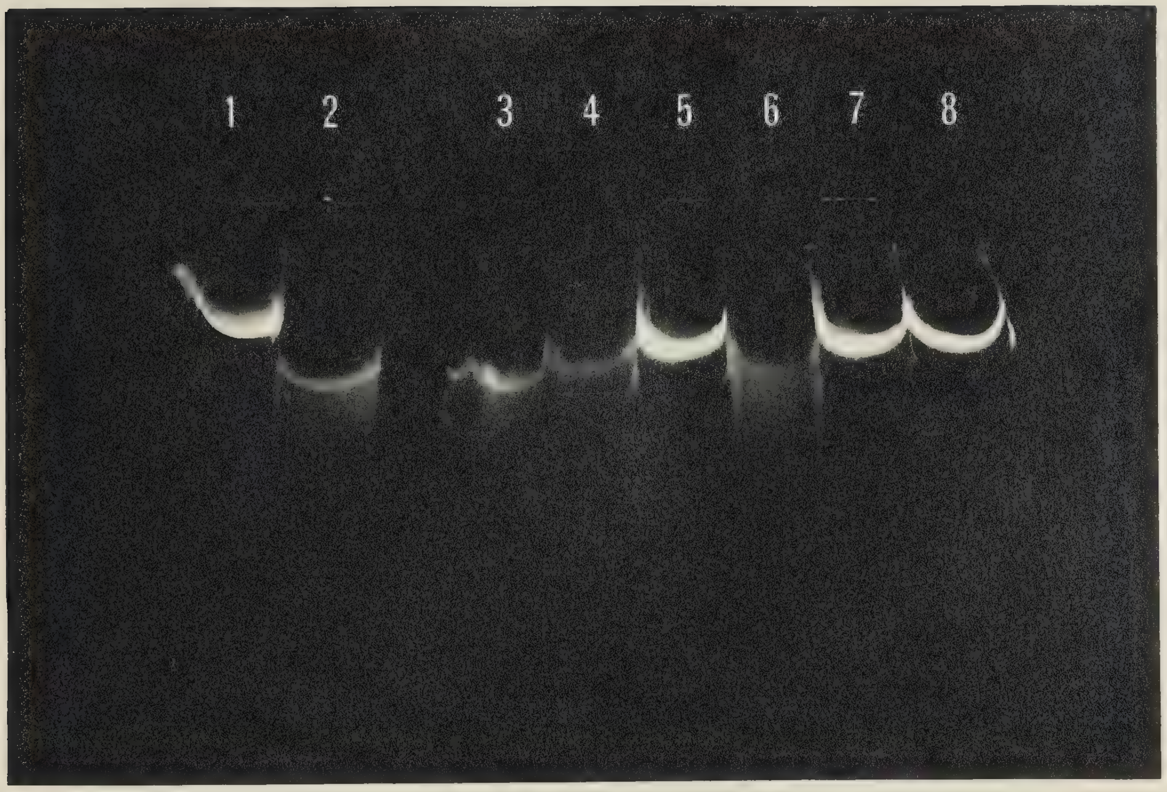

1
2
3
4
5
6
7
8

Fig. 27

Agarose gel electrophoresis:

Cleavage pattern of HAd5 DNA by AAEL-activity protein before and after Eco RI restriction enzyme cleavage.

1: Control

2: Eco RI

3: Eco RI followed by SCP1 (Fig. 12)

4: SCP1 followed by Eco RI

5: Eco RI followed by SCP2 (Fig. 11)

6: SCP2 followed by Eco RI

7: VP1 (Fig. 3)

HAd5 DNA was used as substrate DNA.

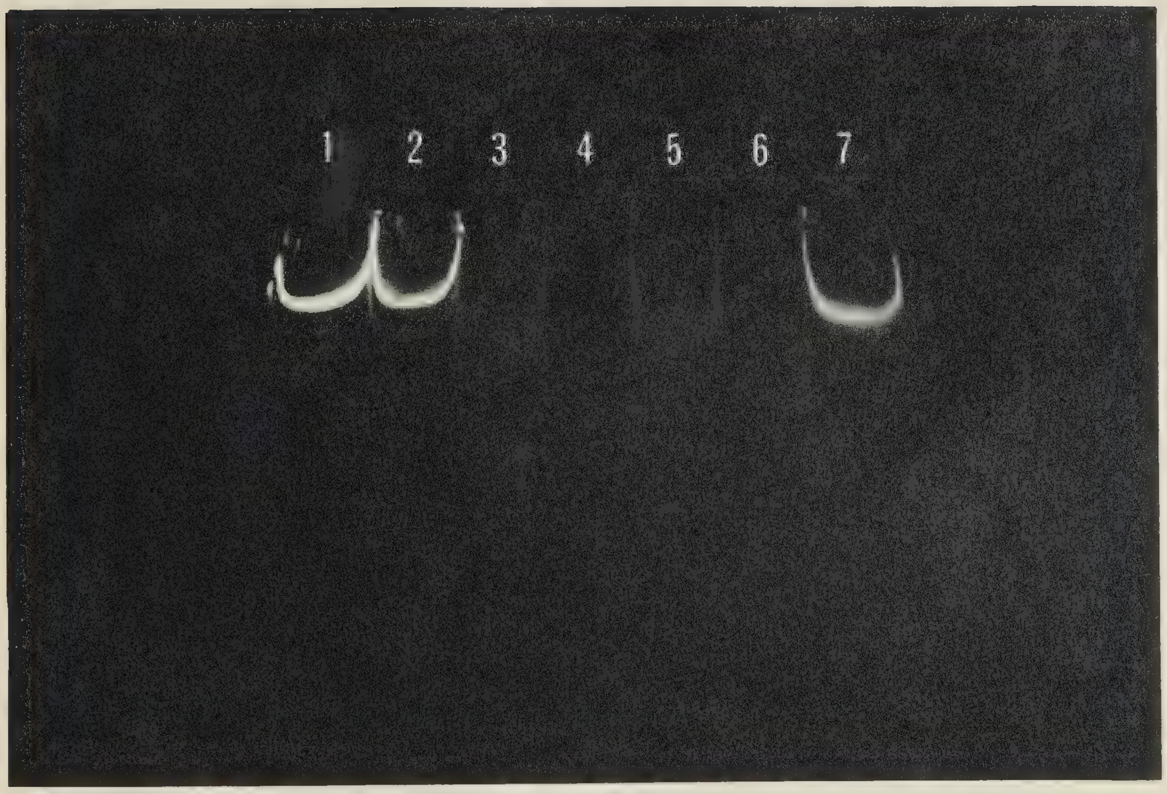

1
2
3
4
5
6
7

Fig. 28

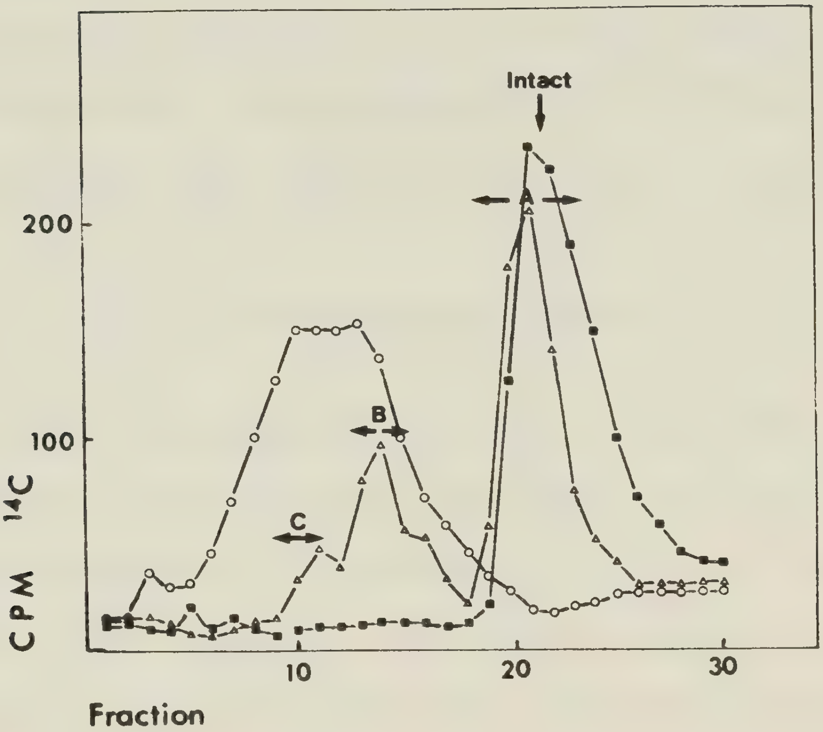
Separation of Eco RI restriction enzyme-derived fragments and SCP1-derived (Fig. 12) fragments of HAd5 DNA.

Details of the preparation are described in Materials and Methods. Samples were layered onto a 0-30% sucrose gradient above a 60% cushion and centrifuged at 80,000 xg for 17 h. Contents of the tube were collected and ^{14}C activity determined in a scintillation counter.

△-△ : Eco RI restriction fragments

o-o : Fragments of HAd5 after SCP1 cleavage

□-□ : Intact HAd5 DNA


Intact
A
B
C
200
100
CPM ¹⁴C
10
20
30
Fraction

Intact HAd5 DNA and Eco RI fragments of HAd5 DNA were centrifuged simultaneously for comparison of fragment sizes post-P1 cleavage.

The distribution of ^{14}C labelled HAd5 DNA after 30 min incubation with SCP1 indicated that there was a population of different sizes of fragments similar to EcoRI fragments B, C and smaller. This also supported the observation of the smearing effect in agarose gels after SCP1 cleavage (Fig. 26).

5 Iodination of AAEL-activity protein.

Purified SCP1 isolated from SC, (Fig. 12) was labeled with ^{125}I and reacted for 1 min with ^{14}C DNA then subjected to centrifugation at 40,000 rpm for 64 h. The result is illustrated in Fig. 29. ^{125}I counts remained at the top of the gradient even after 64 h centrifugation, but the control DNA and protein-treated HAd5 DNA appeared as a sharp peak. This indicated, therefore, that the protein did not bind to the DNA and/or that 1 min incubation of protein with HAd5 DNA was not sufficient to cleave the DNA into a population of different sizes. Release of a small fragment from the intact substrate molecule would not cause an observable change.

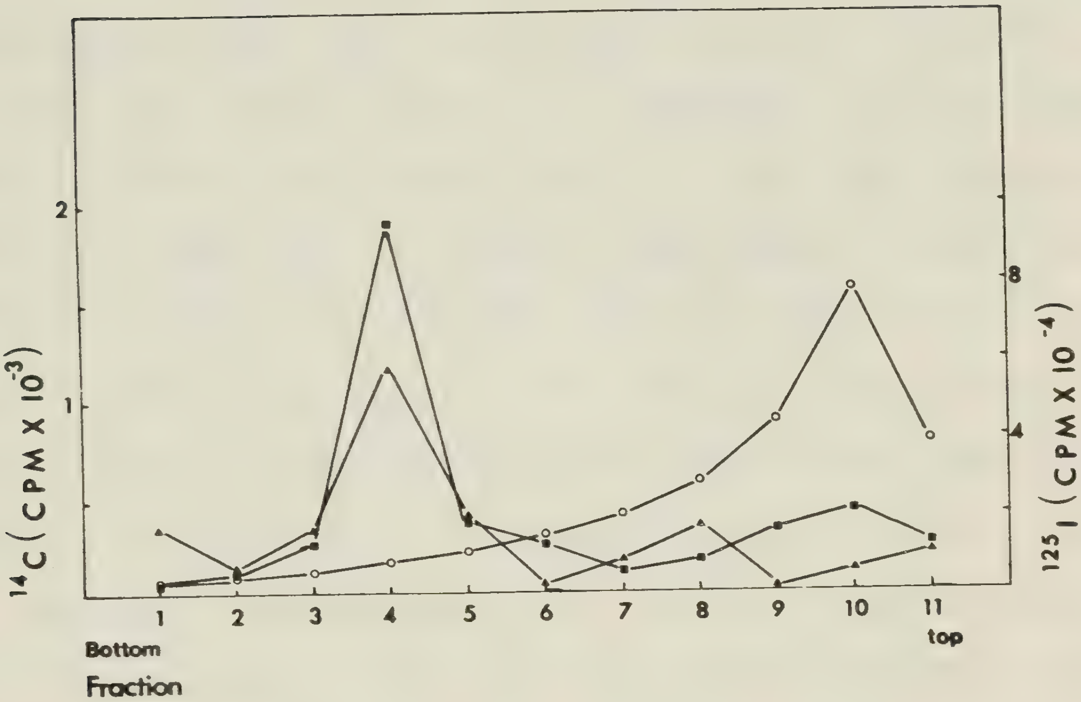
Fig. 29

Profile of ^{14}C HAd5 DNA and ^{125}I SCP1 following equilibrium centrifugation in 4 M guanidine HCL-3 M CsCl solution.

^{125}I SCP1 was reacted with HAd5 ^{14}C DNA for 1 min and layered on to a solution of 3 M CsCl in 4 M guanidine HCL and centrifugated at 100,000 xg for 64 h. Fractions were collected and counted.

○ : Control

■ , ▲ : Test



^{14}C (CPM X 10^{3})
2
1
^{125}I (CPM X 10^{-4})
8
4
1
2
3
4
5
6
7
8
9
10
11
Bottom
top
Fraction

6 Analytical centrifugation of AAEL-activity.

The same type of reaction as described in the iodination experiments was carried out in this part of the study. In Fig. 30, frame 1, the photographs were taken after 23.5 h centrifugation at 44,000 rpm. Both DNA bands appeared distinctly at the same distance from the meniscus. The gradient was then relaxed by lowering the centrifugation speed to 30,000 rpm (frame 2 and 3). If any protein was bound to the DNA, a shift in the density of DNA would be observed. Initial observation indicated that there was no density shift in reacted DNA and also that there was an excess amount of DNA. Therefore 0.2 ml of the solution was removed and 0.25 ml of 4 M quanidine HCl, 3 M CsCl solution was added and equilibrated by further centrifugation at 44,000 rpm for 23 h. In the test, a protein band was observed which was missing in the control (Fig. 30 1-4). The DNA band was observed at the same distance away from the meniscus in both control and test.

7 Detection of AAEL-activity in ts-muntants and recombinants.

HAd2 mutants, ts 1, ts 3, ts 4, ts 48 and HAd5 ts36

Fig. 30

Analytical centrifugation of HAd5 SC derived AAEL-activity protein and HAd5 DNA.

AAEL-activity protein: SCP1 from Fig. 12.

Details of the experiment are described in Materials and Methods.

Frame A: Picture were taken at 30, 40, 60 and 80 sec. intervals following centrifugation at 44,000 rpm for 23.5 h.

1-4 : Test with HAd5 DNA and SCP1
5-8 : Control with HAd5 DNA

Frame B: The CsCl gradient was relaxed by centrifugation at 30,000 rpm. Pictures were taken at different times after relaxation.

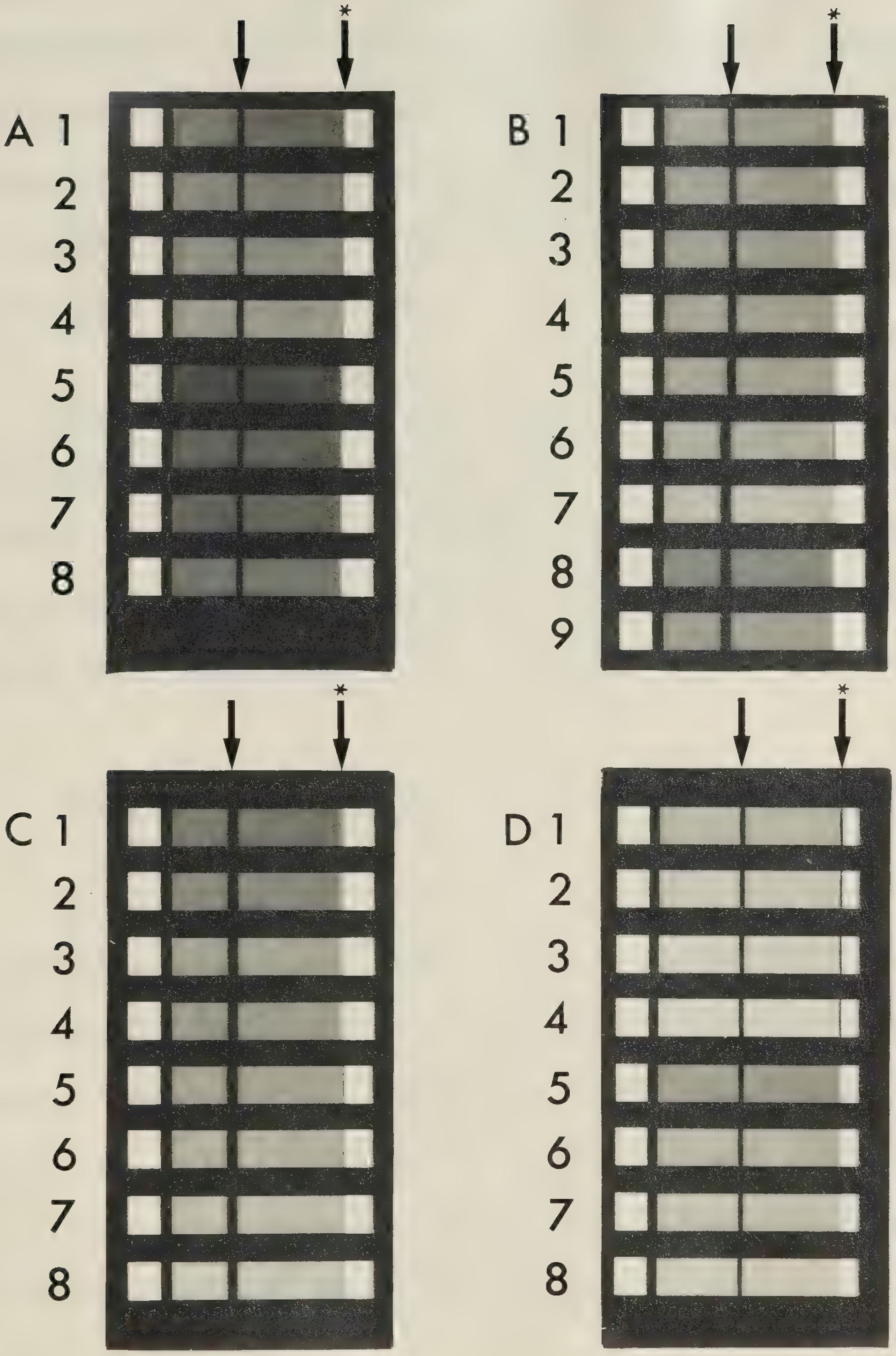
1-7 : Test sample 22.5 post relaxation
8-9 : Controls

Frame C: Pictures taken at 90, 120, 150 and 180 sec intervals following 22.5 h post relaxation.

1-4 : Control
5-8 : Test

Frame D: 0.2 ml of solution was added and 0.25 ml guanidine HCL- CsCl solution added. Pictures were taken 60, 90, 120, 150 sec. intervals following 23 h configuration at 44,000.

1-4 : Test
5-8 : Control
↓ HAd5 DNA
*↓ protein


*
A
1
2
3
4
5
6
7
8
*
B
1
2
3
4
5
6
7
8
9
*
C
1
2
3
4
5
6
7
8
*
D
1
2
3
4
5
6
7
8

grown at 33°C and 39°C (permissive and non-permissive temperature) were tested for AAEL-activity associated with their soluble components . The results summarized in Table 4 indicate that all mutants exhibit AAEL-activity regardless of whether extracts were from permissive or non-permissive incubation temperatures.

Table 5 shows that all adenovirus recombinants were positive for AAEL-activity. Penton-base antiserum and penton antiserum specifically blocked recombined adenovirus AAEL-activities. Virion-specific antisera obtained from Dr. J. Weber from Sherbrooke also blocked AAEL-activity.

8 Endonuclease activity from calf serum and HAd5 .

Calf serum at 10^{-4} dilution displayed comparable endonuclease-like activity to the high speed centrifugation supernatant of disrupted HAd5 grown in the absence of calf serum. (Fig. 31). Since the virus was prepared from cells grown in the absence of calf serum, there was no possibility, therefore, that this endonuclease-like activity was a contaminant from calf serum.

Table 4

Detection of AAEL-activity by spectrofluorimetric assay of soluble components of HAd2 and HAd5. ts muntant grown at permissive and non-permissive temperatures.

	PRESENCE OF AAEL-ACTIVITY	
	33°C	39°C
MUTANT		
HAd2 ts1	+	+
HAd2 ts3	+	+
HAd2 ts4	+	+
HAd2 ts48	+	+
HAd5 ts36	+	+

Table 5

Blocking effect of various antisera on AAEL-activity of different adenovirus ts mutants and recombinants.

AAEL-activity was detected by spectrofluorimetric assay.

KS 115 : Antiserum versus penton base

KS 347 : Antiserum versus penton

recomb : Recombinant

con : control with no antiserum

XHAd2
XHAd5
— antisera obtained from Dr. J. Weber.

1 : ΔFU

2 : % blockage

nd : not done

RECOMB	ANTISERA								
	ΔFU					%			
	CON	KS115	KS347	XHAd2	XHad5	KS115	KS347	XHAd2	XHAd5
8- 2	12.0[1]	0.0	0.0	nd	nd	100[2]	100	nd	nd
2-15	17.5	6.0	1.5	0.0	0.0	65.1	91.4	100	100
4- 9	20.0	2.5	3.0	0.0	1.0	87.5	85.0	100	95.0
4-21	18.5	3.5	3.0	0.0	0.5	81.0	83.8	100	97.3
6- 4	17.0	3.0	1.5	0.0	1.5	82.4	91.2	100	91.2
HAd5	19.0	0.0	0.0	0.0	0.0	100	100	100	100

Fig. 31

Detection of endonuclease-like activity in calf serum and HAd5-derived 80,000 xg supernatant.

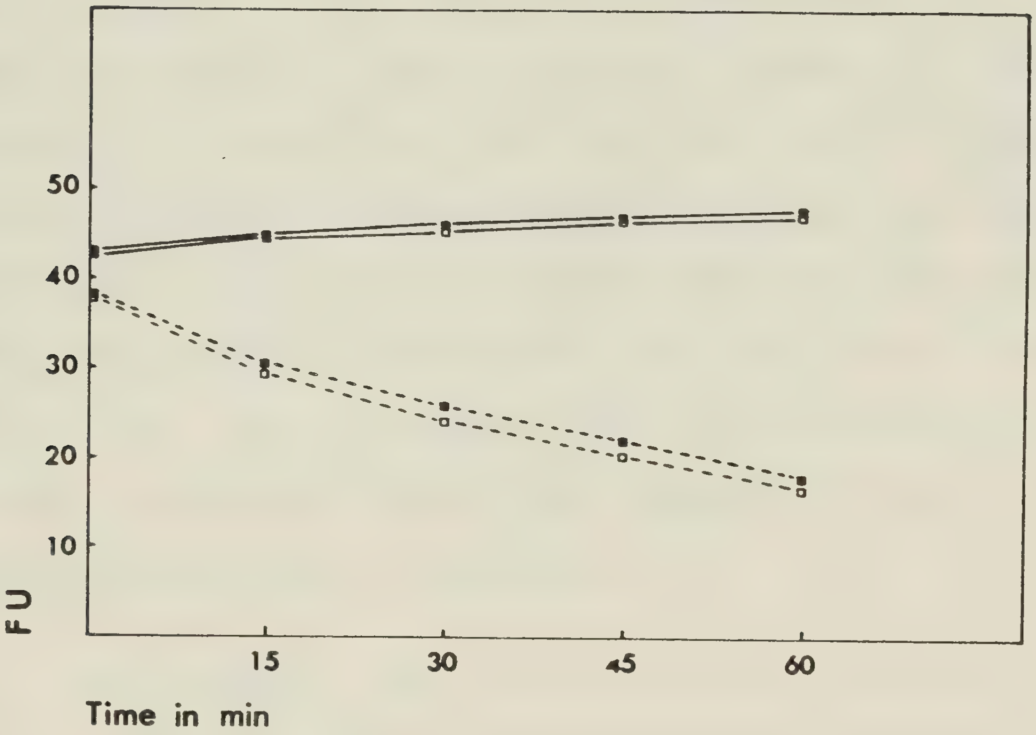
■ 80,000 xg supernatant.

□ calf serum, 10^{-4} dilution.

— Before heating.

-- After heating.

PM2 DNA was utilized as substrate for the detection of endonuclease-like activity.


50
40
30
20
10
FU
15
30
45
60
Time in min

DISCUSSION

In this study of the purification of an endonuclease-like activity in association with disrupted virions or SC (soluble components) material from HAd5, one type of protein was detected which existed in different forms with respect to changes in physical conditions. A schematic representation of the different forms of AAEL-activity protein is shown in Fig. 32. Both forms of AAEL-activity were present in disrupted virions or in SC material. The VP1 (virion-derived) or SCP1 (SC-derived) material which eluted from chromatography columns in the void volume was suspected to be in an aggregated form. This phenomenon might be a result of dialysis versus TMB (change in pH) during the completion of virus disruption.

The second form of AAEL-activity consisted of protein associated with pentons, as indicated by positive HE tests. When high speed supernatant (80,000 xg) from disrupted HAd5 virions was further purified by cation-exchange chromatography, both free and penton-associated protein eluted in the void volume as a single peak, but when anion-exchange chromatography was utilized, free AAEL-activity, in an apparent aggregated form, was eluted in the column void volume, probably as a result of the difference in the net

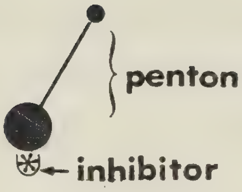
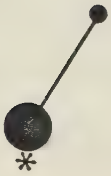
Fig. 32

Different forms of SC or virion derived AAEL-activity HAd5.

Blocking protein : AAEL-activity protein with associated inhibitor.

AAEL-act. protein I : AAEL-activity protein associated with intact pentons.

AAEL-act. protein II: AAEL-activity protein in free aggregated form.

		ACTIVITIES		SOURCE
		HE	AAEL	
Blocking protein		+	−	80,000Xg
AAEL-act. protein I		+	+	VP_2 SC_2
AAEL-act. protein II		−	+	VP_1 SC_1

ionic charge on the surface. The penton-associated AAEL-activity eluted at the beginning of the salt gradient (Fig. 2), indicating that the pentons were positively charged. With Affi-Gel Blue chromatography, a similar profile was obtained and VP1 was again eluted in the void volume.

An identical chromatographic profile was obtained with SC material in anionic-exchange chromatography (DEAE-Sephadex A50). SCP1 (Fig. 11) eluted in the void volume, as did SCP2 after concentration and dialysis against TMB, and was further purified by Affi-Gel Blue chromatography. A peak of protein with decreased HE activity was obtained in the void volume. From this observation, it was apparent that aggregation of AAEL-activity protein had occurred and the protein had become free from penton as indicated by the large reduction in HE titer (indicating the absence of penton monomers).

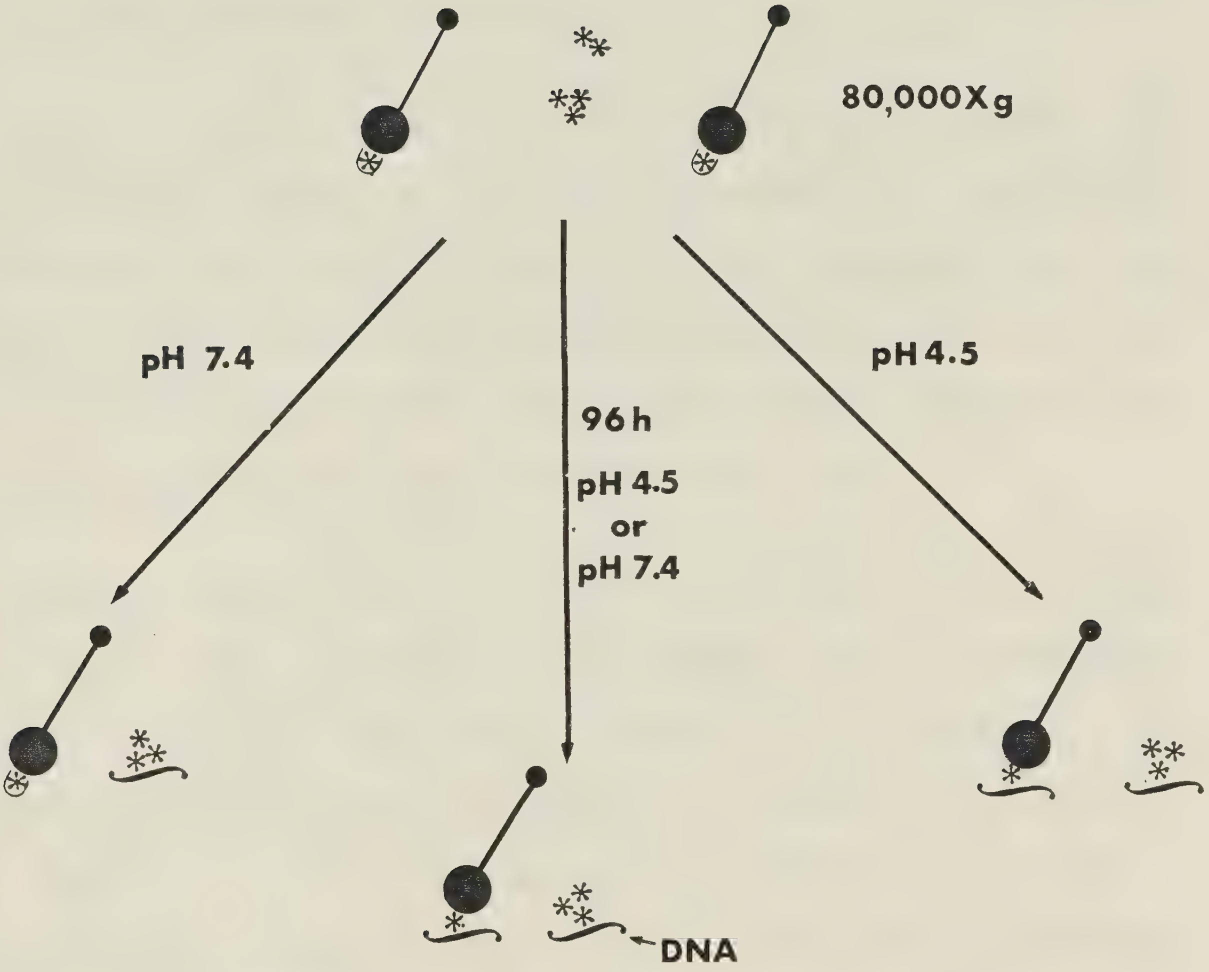
PAGE analysis revealed that all four forms of the protein, VP1, VP2, SCP1, and SCP2, had the same molecular weight of 32,000-32,500. According to the findings of Reif et al. (1977 a,b), purified endonuclease from HAd2-infected KB cells had a molecular weight of 41-42,000, as determined by gel filtration on Biogel A-0.5m, 48,000 on glycerol gradients and 43-44,000 in SDS-polyacrylamide slab gels. A

protein with a molecular weight of 32-32,500 should be retained in a Sephadex G100 exclusion gel, but contrary to expected results, the protein was eluted in the void volume. This finding further suggested that protein aggregation had occurred.

The fluorimetric assay for detection of AAEL-activity in the high speed (80,000 Xg) supernatant utilized two buffers with different pHs in the incubation mixture. Only minute amounts of activity were detected when pH 7.4 buffer was used in the incubation mixture in contrast to the high level of activity detected when pH 4.5 buffer was used. After storage of the 80,000 xg supernatant at room temperature for 96 h, there was an increase in the amount of pH 7.4 activity, equivalent to the amount detected with pH 4.5 buffer before storage. This observation led to the following interpretation (Fig. 33). In pH 7.4 buffer, only the aggregated form of AAEL-activity protein actively cleaved the substrate DNA. Penton-associated AAEL-activity is complexed with an inhibitor, as also described by Reif et al.(1977 a,b), or a factor, protein in nature, blocking the enzymatic site, and activity was therefore not detected. In pH 4.5 buffer, the inhibitor becomes unstable and detaches from the AAEL-activity, making both forms of the

Fig. 33

Effect of pH on AAEL-activity in the presence of blocking-protein.


80,000Xg
pH 7.4
96 h
pH 4.5
or
pH 7.4
pH 4.5
DNA

protein available for cleavage of substrate DNA and resulting in an increase in amount of activity as detected in the fluorimetric assay. The data indicate, therefore, that this inhibitor or blocking factor is unstable at low pH and at room temperature after 96 h.

During the first step of purification by exclusion gel chromatography, AAEL-activity was detected in association with penton. This would indicate that the inhibitor was most probably removed during the purification step. This was also shown in the purification of an endonuclease isolated from HAd2-infected KB cells (Reif et al., 1977 a,b).

Pronase (200 ug/ml final concentration) has been shown to remove the inhibitor associated with endonuclease activity isolated from HAd2-infected KB cells, thus resulting in an increase in enzymatic activity (Reif et al., 1977 a,b). In this investigation of Pronase treatment of AAEL-activiy, a concentration of 200 ug/ml and incubation for 2.5 h at 37°C did not affect the AAEL-activity. The inhibitor was not degraded by Pronase treatment as no increase in activity was detected. Following overnight incubation at 37°C of the Pronase-AAEL-activity protein, no AAEL-activity was detected.

In other viruses, eg., polyoma and SV40, mercaptoethanol and detergents such as sodium deoxycholate have been utilized for the removal of inhibitors or unmasking of an enzyme in order to enchance its activity (Pogo & Dales, 1969; Kang and McAuslan 1972; Rouget et al., 1976). The AAEL-activity protein was stable at room temperature but not at 4°C or -20°C. Purified AAEL-activity protein from column chromatography, stored at room temperature, has remained active for nine months (thus far detected).

As there was an ion requirement for AAEL-activity, the addition of a chelating agent such as EDTA, inhibited the reaction. At concentrations of less than 5%, SDS did not affect the AAEL-activity. At a concentration of greater than 5% SDS, no AAEL-activity was detected indicating that the enzymatic sites might have been destroyed.

Besides affecting the AAEL-activity inhibitor, pH most likely also causes differences in the conformational state of the AAEL-activity protein purified from gel chromatography, as indicated by mobility profiles of cleaved substrate DNA on agarose gels. With PM2 or HAd5 DNA as substrate DNA at pH 4.5, the cleavage observed appeared not to be as efficient as in pH 7.4 buffer (Fig. 23, 26).

Results from PAGE analysis revealed that AAEL-activity protein derived from disrupted virions or SC material of HAd5 had the same molecular weight (as discussed above). This clearly indicates, therefore, the existence of one AAEL-activity protein having different conformational states and cleavage patterns under altered physical conditions, particulary pH. Endonuclease activity having different pH optima due to conformational changes was also observed by other investigators (Reif et al., 1977 a,b).

Results obtained in the DNA cleavage pattern study indicated that AAEL-activity protein might recognize a specific substrate site. With PM2 DNA substrate, two bands were obtained in agarose gel electrophoresis. The highest molecular weight population migrated at the same rate as the control DNA, indicating the presence of excess DNA in the assay. The lower molecular weight population in the gel indicated that there was a population of cleaved PM2 DNA. An increase in VP1 protein increased the intensity of the lower band, indicating that the cleavage was dependent on the concentration of the protein. SCP1, SCP2 and VP2 cleaved PM2 DNA and produced a smear on the agarose gels, again indicating that the degree of substrate cleavage was proportional to the amount of AAEL-activity protein present.

HAd5 or HAd2 DNA was cleaved by SCP1, SCP2 and VP2 into different sized fragments. In this study of the cleavage site specificity of the AAEL-activity protein, the data suggested that the protein recognized a site close to the 3' or 5' end, possibly close to the origin of replication, and cleaved the subtrate DNA at that site as initially indicated by a slight smearing of the DNA band in agarose gels (as compared to control DNA after short time incubation; 90 sec; Fig. 34). With longer incubation periods, more of the specific sequence was apparently recognized and cleaved, consequently resulting in the further smearing of the DNA band (composed of a population of different sizes of DNA; Fig. 34). This finding was further supported by sucrose gradient centrifugation of the cleavage products of HAd5 DNA with SCP1. In centrifuging intact HAd5 DNA and HAd5 Eco R1 restriction fragments A, B and C in sucrose gradients, the profile of the DNA distribution obtained (Fig. 28) indicated that the fragments resulting from SCP1 cleavage had sizes varying from greater than B to smaller than C, co-inciding with the smearing of the DNA band observed in agarose gels (Fig. 26).

Analysis of the cleavage product of an endonuclease from HAd2-infected KB cells (Reif et al., 1977 a,b) by

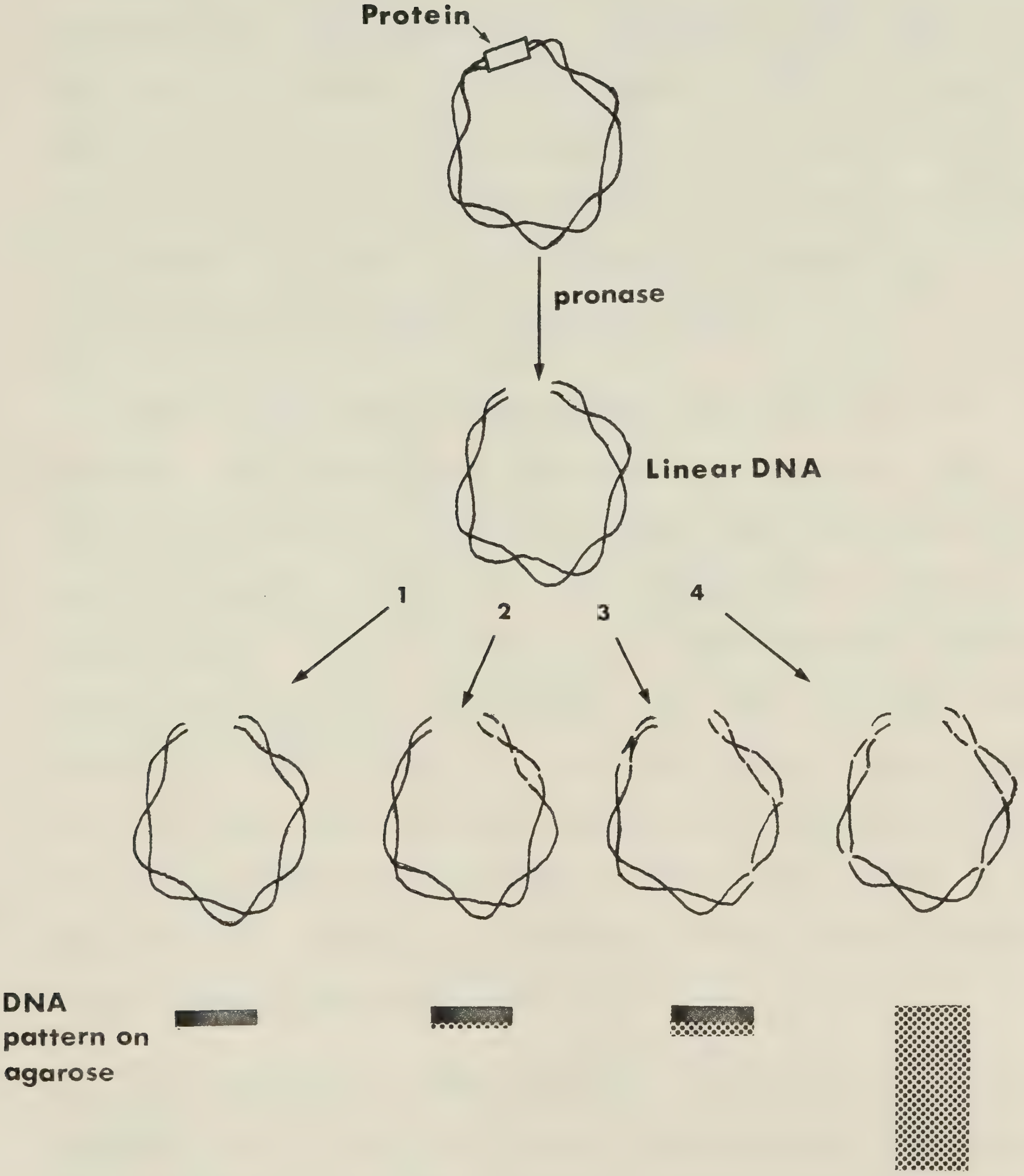
Fig. 34

Schematic representation of the relationship of AAEL-activity HAd5 substrate DNA cleavage and banding patterns in agarose gel electrophoresis.

1 : intact HAd5 DNA without AAEL-activity protein

2 & 3 : initial cleavage

4 : Prolonged incubation of AAEL-activity
with HAd5 DNA


Protein
pronase
Linear DNA
1
2
3
4
DNA pattern on agarose

velocity sedimentation, sucrose density gradient or gel electrophoresis on composite polyacrylamide/agarose gels, indicated that the size of DNA fragments decreased successively with prolonged incubation time, but the size did not go below 5-6 S fragments. From sedimentation analysis, and gel electrophoresis, the size distribution was heterogeneous. It was suggested that the enzyme recognized a nucleotide sequence which occured frequently near the G-C rich region (Burlingham & Doerfler, 1971).

Since the AAEL-activity has an affinity for viral protein, the penton base (Burlingham & Doerfler, 1971), and does form aggregates, the possibility that the AAEL-activity protein might also be a DNA-binding protein was investigated. From the results of iodination and analytical centrifugation data, indications were that the AAEL-activity protein did not bind to DNA and cause endonucleolytic cleavage as observed with SV40 (Kasmatsu and Wu, 1976), or the protein-DNA complex found in association with E. coli plasmid DNA (Blair & Helinski, 1975; Lovett & Helinski, 1975; Guiney & Helinski 1975).

From studies of SV40 mutants (Dooley et al., 1971), including one of the ts A class, endonucleases were detected at non-permissive temperature and no heat labile

endonuclease was found in several ts-mutants of SV40 which logically might code for an endonuclease.

Cell extracts of temperature-sensitive (ts) conditional lethal mutants grown at permissive and non-permissive temperatures were positive for AAEL-activity as detected by fluorimetric assay. HAd2 ts1, HAd2 ts3, HAd2 ts4, HAd2 ts48, the assembling mutants were positive for AAEL-activity at non-permissive temperature suggesting that there was no apparent relationship between the AAEL-activity protein and missing polypeptides in these mutants.

HAd5 ts36, a DNA minus mutant, was positive for AAEL-activity at non-permissive temperature. Since protein synthesis does not occur at non-permissive temperature in this mutant, this suggested the pre-existence of protein with AAEL-activity.

All the recombinants tested were positive for AAEL-activity and the activity could be blocked by antisera against HAd2 and HAd5. Furthermore AAEL-activity has been found in association with non-infected cells (this study; Reif et al., 1977 a,b), strongly suggesting the pre-existence of AAEL-activity protein (Burlingham et al., 1972). If this AAEL-activity protein was host cell-

specified, the differences in reported molecular weight between this study and that of Reif et al. (1977 a,b) might be accounted for by the fact that different virus-host-cell systems were used: infected HAd2 KB cells in Reif's study and HAd5-infected HEp-2 cells in this study.

Strong evidence has been presented that the AAEL-activity protein is host cell-specified, viral-induced, viral-modified and incorporated into virions. In the study by Medveczky et al. (1976), host cell antiserum did not inhibit the endonuclease-like activity. In this study, antiserum against purified components of uninfected cells showed partial blockage of the AAEL-activity and antisera against disrupted virions or purified subunit (penton or penton base) inhibited 100% of the AAEL-activity. AAEL-activity detected from ts- mutants was also blocked to a large extent as shown on Table 5. The evidence therefore strongly suggests that the AAEL-activity protein originates from host cells. The AAEL-activity is not a contaminant from calf serum, as suggested by various investigators (McMillen et al., 1976; Dooley et al., 1976), as virus prepared from calf serum-free cells also showed AAEL-activity and calf serum endonuclease has different properties (Reif et al., 1977 a,b).

The exact function of the AAEL-activity protein is unknown. There is a possibility that it initiates a single strand "nick" close to the origin of replication. Another function might be the "nicking" of several sites during the formation of recombinants between viral and cellular DNA molecules. Such a role for viral-association endonuclease had been suggested by various investigators (Michel et al., 1967; Stocker & Dulbecco, 1969; Cuzin et al., 1970; Parodi et al., 1974.)

An AAEL-activity protein isolated and purified from HEp2 cells infected by HAd5 probably is identical to the endonuclease reported by Reif et al. (1977 a,b) in association with HAd2 or the endonuclease found in association with HAd1 (Medveczky et al., 1976).

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B30220